Two-Dimensional Pulsed EPR Spectroscopy of the Copper Protein Azurin

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Received August 9, 1995[⊗]

Abstract: Two-dimensional (2D) pulsed EPR spectroscopy was applied to study the copper ligands in azurins from *Pseudomonas aeruginosa*, Az(pae), and *Alcaligenes species NC1IB 11015*, Az(asp), in frozen solutions. While a high-resolution three-dimensional crystal structure is available for Az(pae), only a low-resolution structure has been reported for Az(asp). Az(pae) was studied in the pH range 3.9−7.0 and Az(asp) at a pH of 4.8. Measurements were performed at 9 GHz which is usually within the cancellation condition for the remote nitrogen of imidazole ligands. The main technique was the hyperfine sublevel correlation (HYSCORE) technique. At all pH values investigated the 2D HYSCORE spectra of Az(pae) showed correlations between the nuclear frequencies corresponding to the nuclear quadrupole resonance (NQR) frequencies of the remote nitrogens of the imidazole ligands and the double quantum frequency. The spectra showed additional well-resolved cross peaks which indicate correlations between the NQR frequencies of a weakly coupled amide nitrogen and the corresponding double quantum frequency. This confirms earlier detection and assignment of the electron spin−echo envelope modulation (ESEEM) frequencies of this nitrogen which were based on ESEEM measurements of the H117G mutant (Coremans *et al. Chem. Phys. Lett.* **1995**, *235*, 202). The 2D spectra of Az(asp) were similar to those of Az(pae) showing that a third weakly coupled nitrogen is present in this species as well. HYSCORE spectra of a frozen solution of ascorbate oxidase exhibited only signals corresponding to the remote nitrogens of the imidazole. Comparing these spectra with those of the azurins and correlating the results with the available crystal structures of ascorbate oxidase and Az(pae) suggest that the third nitrogen in Az(pae) is the amide nitrogen of His-46, coupled to the copper *via* the carbonyl group of Gly-45. This further implies that also in azurin from Az(asp), the precise 3D structure of which is not yet available, the copper has five ligands rather than four. This study demonstrates that the 2D HYSCORE experiment is most useful for detecting, unraveling, and assigning ESEEM frequencies in metalloproteins.

Introduction

Azurin is a blue single copper protein functioning as an electron carrier in bacterial redox chains.[1] All members of the blue copper proteins family contain a type I copper site which has unique spectroscopic properties; an intense blue color due to a S(Cys) → Cu(II) charge transfer absorption near 600 nm ($\epsilon \approx 5000\ M^{-1}\ cm^{-1}$) and a relatively small Cu(II) hyperfine interaction ($A_{||} \leq 90 \times 10^{-4}\ cm^{-1}$).[2,3] The available three-dimensional structures of several copper proteins with a type I site reveal three strong ligands, a cysteine thiolate and two imidazoles residues of histidine, in an trigonal arrangement with rather short bonds (≈2 Å) and a fourth, weaker axial ligand (usually a thioether of methionine) at a distance of ≈3 Å.[4−7] In stellacyanin the thioether is supposedly replaced with the glutamine amide as the axial ligand.[8,9] Azurin is exceptional in the sense that crystal structure shows an additional weak axial copper interaction with a peptide carbonyl from glycine-45 at a distance around 3 Å (see Figure 1a).[4,5] The actual coordination to this oxygen has, however, not yet been unambiguously established. This issue is important since modulation of the Cu(II)/Cu(I) redox potential has been attributed to the nature of the axial ligands and the number and strength of the NH···S-(Cys) hydrogen bonds.[10−12]

A recent electron nuclear double resonance (ENDOR) study of a single crystal of azurin from *Pseudomonas aeruginosa* at 95 GHz and three-pulse electron spin−echo envelope modulation (ESEEM) of the frozen solution of the H117G mutant at 9 GHz have assigned the magnetic tensors of the remote nitrogens in the imidazole ligands. Moreover, a third weakly coupled nitrogen has been detected and was assigned to a backbone amide nitrogen. The candidates proposed for this nitrogen were the backbone nitrogens of either Cys-112 or His-46 which is conjugated to the carbonyl of Gly-45.[13] If the latter is the case, then these results provide unambiguous evidence

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[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.

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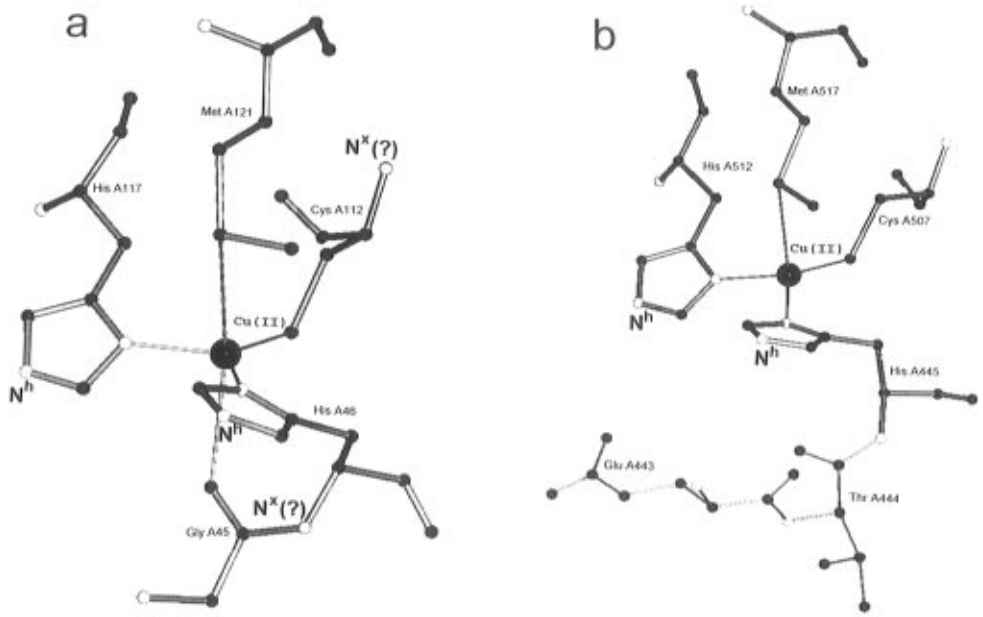

Figure 1. The 3D structure of the copper type I site of (a) Az(pae)[5] and (b) AO.[6] N^h and N^x correspond to the remote nitrogen of the imidazole and the nitrogen which may be the third weakly coupled nitrogen, respectively.

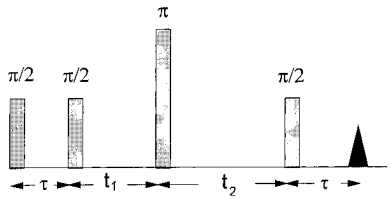

Figure 2. The HYSCORE pulse sequence.

for the existence of a weak covalent bond between the Cu(II) and the Gly-45 carbonyl.

In this work we show that ESEEM signals of the third weakly coupled nitrogen in native azurin in a frozen solution are readily detected by two-dimensional (2D) hyperfine sublevel correlation (HYSCORE) spectroscopy. Moreover, the cross peaks observed in the two-dimensional HYSCORE spectra facilitate significantly the assignment of these ESEEM frequencies.

HYSCORE is a 2D four-pulse ESEEM experiment which provides correlations between ESEEM frequencies belonging to different M_S manifolds.[14] It has recently been proven to be most useful for the assignment of ESEEM signals[15,16] and for the detection of broad signals which are absent from the ESEEM spectrum due to the spectrometer's dead time.[16,17] The pulse sequence of the HYSCORE experiment is shown in Figure 2. It is derived from the three-pulse echo sequence (stimulated echo) where a π pulse has been introduced in between the last two $\pi/2$ pulses. The first two $\pi/2$ pulses, separated by the time interval τ, represent the preparation period which generates nuclear coherences.[18] These coherences evolve during the evolution time t_1 and the π pulse, which represents the mixing period, transfers electron spin populations from one M_S manifold to the other. This transfer is accompanied by the mixing of all nuclear coherences in one M_S manifold with all those of the other manifold. During the evolution time t_2, the nuclear coherences continue to evolve, but with their new frequencies. The last $\pi/2$ pulse and the τ interval comprise the detection period after which an echo is formed. The echo intensity is measured as a function of t_1 and t_2 and a two-dimensional Fourier transform yields a 2D spectrum which consists of cross peaks between the ESEEM (ENDOR) frequencies of the $M_S = {}^1/_2$ and $-{}^1/_2$ manifolds.

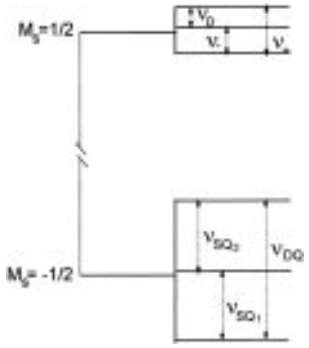

Figure 3. The energy level diagram of $S = {}^1/_2$, $I = 1$ under the condition of exact cancellation.

A schematic energy level diagram for a Cu(II), $S = {}^1/_2$, coupled to one ^{14}N nucleus, $I = 1$, ignoring the copper hyperfine interaction, is shown in Figure 3. This diagram represents a special case where the isotropic hyperfine interaction, A_{iso}, is approximately twice the nuclear Larmor frequency, namely $|A_{iso}| \approx 2\nu_N$. The latter is referred to as the cancellation condition. For the remote nitrogen in Cu(II) complexes, with imodazole it is met at ~9 GHz.[19,20] When this condition is fulfilled the effective field experienced by the nucleus in one of the $M_S$manifolds is approximately zero. The ESEEM frequencies within this manifold are therefore close to the ^{14}N nuclear quadrupole resonance (NQR) frequencies, ν_0, ν_-, and ν_+. These frequencies appear as relatively narrow, orientation-independent signals in the ESEEM spectrum. The ESEEM frequencies of

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the M_S manifold in which the hyperfine and the Zeeman fields add rather than cancel each other are ν_{SQ_1}, ν_{SQ_2}, and ν_{DQ}. The first two correspond to single quantum nuclear transitions, $\Delta M_I = \pm 1$, whereas the third one corresponds to a double quantum transition, $\Delta M_I = \pm 2$. The ν_{SQ_1} and ν_{SQ_2} signals are usually hard to observe in ESEEM spectra of orientationally disordered systems because they usually decay within the spectrometer deadtime due to their excessive inhomogeneous broadening. The inhomogeneous broadening of ν_{DQ} is significantly smaller than that of ν_{SQ_1} and ν_{SQ_2}, and it usually appears in the ESEEM spectra of frozen solutions.

According to the energy level diagram shown in Figure 3, the 2D HYSCORE spectrum should show 9 cross peaks: (ν_0; ν_{DQ}), (ν_-; ν_{DQ}), (ν_+; ν_{DQ}), (ν_0; ν_{SQ_1}), (ν_-; ν_{SQ_1}), (ν_+; ν_{SQ_1}), (ν_0; ν_{SQ_2}), (ν_-; ν_{SQ_2}), (ν_+; ν_{SQ_2}). In frozen solutions the first three appear as well-resolved narrow peaks whereas the last six show as nonresolved ridges. Exceptions are cases where the HYSCORE spectrum is recorded under orientation selective conditions at an external magnetic field corresponding to the $g_{||}$ position.[16]

HYSCORE measurements were performed at several pH values on azurin isolated from *Pseudomonas aeruginosa*, Az-(pae), for which a high-resolution 3D structure is available[5] and on azurin from *Alcaligenes species NCIB 11015*, Az(asp), formerly known as *Pseudomonas denitrificans*.[21] The structure of Az(asp) has been solved only to a 3.0 Å resolution;[22] His-46, Cys-112, and His-117 were found at a close distance to the copper while Met-121 was observed at a larger distance. Precise bond lengths and angles for the Cu(II) ligands were not quoted and there was no mention of the fifth carbonyl ligand. Nonetheless, amino acid sequences of both proteins show that all five amino acids assumed to serve as copper ligands are conserved.[23] However, since one ligand is the peptide carbonyl of the glycine, it could have been substituted by any amino acid, aside from proline.

Nuclear modulations from the third weakly coupled nitrogen were observed in all azurin solutions investigated. Similar measurements were carried out on ascorbate oxidase (AO) from *Cucurbita pepo medullosa*, and only signals from imidazole nitrogens were observed. Comparison of the AO and azurin 2D spectra suggests that the third nitrogen in azurin is the backbone nitrogen of His-46 connecting Gly-45 rather than that of the Cys-112 backbone.

Experimental Section

Preparation, Purification, and Characterization of the Proteins. *Pseudomonas aeruginosa* and *Alcaligenes species NCIB 11015* bacteria were grown anaerobically for 48 h in a 450-L fermentor. Medium and growth conditions were as described by Rosen *et al.*[24,25] After harvesting, the cells were frozen and stored at −20 °C. The azurins were isolated and purified according to the method of Ambler[26,27] with a slight modification for Az(asp).[25] Az(pae) was eluted at a pH of 3.9 and Az(asp) at a pH of 4.8. Further pH changes for Az(pae) (4.8, 6.0, and 7.0) were obtained by dialysis against an ammonium acetate buffer (0.05 M) of the appropriate pH. The purity of the azurins was assessed by sodium dodecyl sulfate (SDS) gradient polyacrylamide slab gel electrophoresis. It was examined prior to measurements and the optical absorption ratio was $A_{(625/280)} = 0.5$.

AO from *Cucurbita pepo medullosa* was purchased from Boehringer Mannheim and was further purified on a CM-Sephadex C-50 cationic exchange column up to a purity ratio of $A_{(330/612)} = 0.7$.[28] The buffer used for AO was potassium phosphate, 0.1 M, pH = 7.0. The final concentrations of all protein solutions studied were in the range 2−2.5 mM.

Spectroscopic Measurements. The ESEEM and 2D HYSCORE experiments were carried out at 5−8 K at a frequency of ≈9 GHz, using a home-built spectrometer, described elsewhere.[29] The magnetic field and spectrometer frequency were measured with a Bruker NMR Gaussmeter (ER-035M) and a HP-5350B frequency counter. The ESEEM waveforms were recorded using the three-pulse sequence, $\pi/2-\tau-\pi/2-T-\pi/2-\tau-$echo, where the echo intensity is measured as a function of the time interval T. Measurements were carried out with τ values of 170 and 180 ns and a repetition rate of 1 KHz. Typically, the $\pi/2$ pulse length was 20 ns and the increment of T was 20 ns. 2D HYSCORE spectra were recorded using the sequence $\pi/2-\tau-\pi/2-t_1-\pi-t_2-\pi/2-\tau-$echo, where the echo is measured as a function of t_1 and t_2.[14] The duration of the $\pi/2$ and π pulses was 15 ns and the amplitude of the π pulse was twice that of the $\pi/2$ pulses. Between 100 and 140 points were collected in each dimension, the increment of t_1 and t_2 was 40 or 50 ns, and their initial value was 90 ns. The repetition rate was 0.8 kHz and the measurements were carried out at τ values of 300 and 350 ns. The appropriate phase cycles, eliminating unwanted echoes, were employed in all experiments.[30,31]

Data Handling/Analysis. (a) 1D ESEEM: To avoid distortions of the FT-ESEEM spectrum due to spectrometer dead time, which for the three-pulse experiment was typically τ + 40 ns, the missing data points were reconstructed using the linear prediction singular value decomposition (LPSVD) method.[32] Prior to Fourier transformation the background decay was removed either by a polynomial or exponential fit, or by subtracting the appropriate zero-frequency component as obtained from the LPSVD procedure. All spectra shown are the cosine FT-ESEEM. **(b) 2D ESEEM**: The background decay in both t_1 and t_2 dimensions was removed using a third-order polynomial fit and after zero filling to 512 points in each dimension Fourier transform was carried out in the two dimensions. The spectra shown are contour plots in magnitude mode with logarithmic scaling of the contour intervals.

Results

Azurin (*Pseudomonas aeruginosa*). Two-dimensional HYSCORE spectra of Az(pae) at pH values of 3.9, 4.8, 6.0, and 7.0 were measured and Figure 4 shows the spectra obtained at pH 3.9, 4.8, and 7.0. These spectra were recorded at the magnetic field at which the echo intensity reached a maximum, $g \approx 2.06$ ($\approx g_\perp$). The spectra show peaks on the diagonal and several sets of cross peaks. In principle the HYSCORE experiment should not produce any diagonal peaks, in practice they are almost always evident due to incomplete inversion of the magnetization by the π pulse. The peaks on the diagonal of the positive quadrant, (+;+), appear at 0.8, 1.5, 2.0, 2.8, and 3.7−3.9 MHz. This is in a good agreement with previously reported 1D ESEEM results.[13,33] In the following we shall refer to the imidazole remote nitrogens as N^h and to the third nitrogen as N^x. At ≈9 GHz the hyperfine couplings of N^h of His-117 and of N^x are approximately twice the ^{14}N Larmor frequency[13] thus falling within the range where the cancellation condition is met.[19] Therefore, the ESEEM frequencies within one of the

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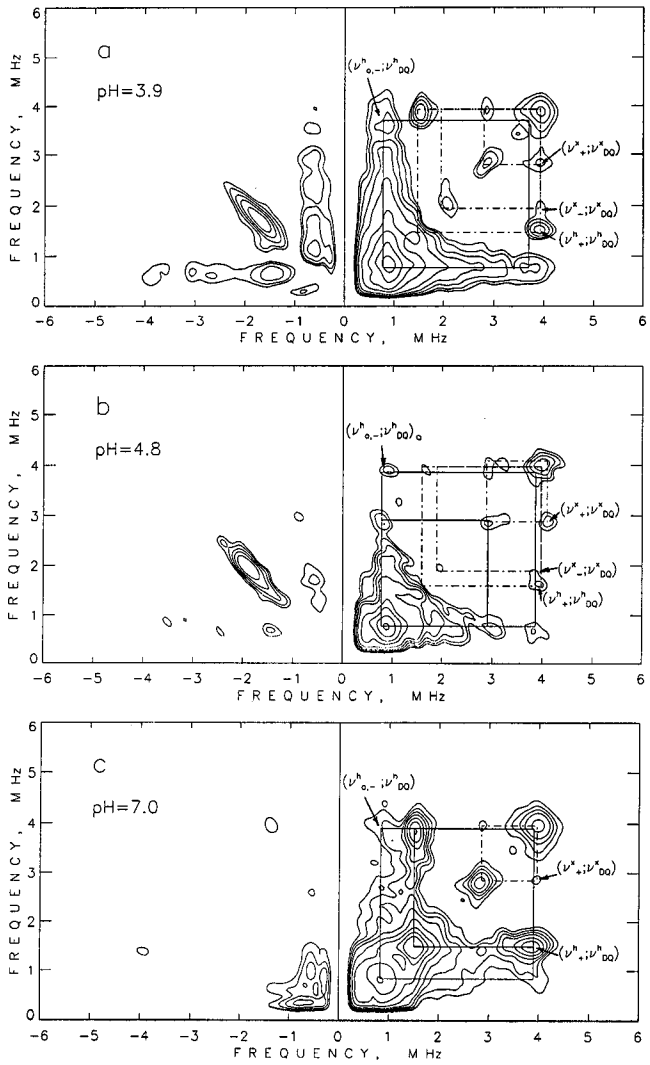

Figure 4. 2D HYSCORE spectra of Az(pae) recorded at $g = 2.06$; (a) pH = 3.9, $\tau = 0.3$ μs, $H_0 = 3115$ G; (b) pH = 4.8, $\tau = 0.3$ μs, $H_0 = 3210$ G; (c) pH = 7.0, $\tau = 0.35$ μs, $H_0 = 3216$ G.

M_S manifolds are ν_0, ν_-, and ν_+. The ν_0 and ν_- signals of the imidazole nitrogens overlap due to the high-asymmetry parameter, $\eta \approx 1$, and in the following it will be referred to as $\nu^h_{0,-}$. The NQR frequencies of N^h of His-117 are 0.8 ($\nu^h_{0,-}$) and 1.50 (ν^h_+) MHz whereas those of the third nitrogen, N^x, are 0.6 (ν^x_0), 2.0 (ν^x_-), and 2.8 (ν^x_+) MHz as assigned in ref 13. Although the diagonal peak at 1.50 MHz is not well resolved, it is evident through its cross peaks (see below). The ν^x_0 peak, expected at 0.6 MHz, is not resolved in these spectra. The isotropic hyperfine of N^h of His-46 is 1 MHz, which is rather far from the values required for efficient cancellation, resulting therefore in a significantly lower modulation amplitude as compared to N^h of His-117.[13] Thus, the majority of the N^h signals are due to His-117, though some overlapping contributions from His-46 are expected. The ν_{DQ} peak of N^h of His-117 and N^x are significantly broader than the NQR peaks and both appear around 4 MHz.

According to the above assignments, the cross peaks of each of the N^h are expected at ($\nu^h_{0,-}$; ν^h_{DQ}) and (ν^h_+; ν^h_{DQ}) and overlap is expected if their double quantum frequencies are not sufficiently different. Similarly, the cross peaks of the third nitrogen should appear at (ν^x_0; ν^x_{DQ}), (ν^x_-; ν^x_{DQ}), and (ν^x_+; ν^x_{DQ}). Table 1 summarizes all the cross peaks observed and their assignments. The position of the cross peaks of the NQR signals of N^h with the corresponding ν_{DQ} indicates a slight shift in ν_{DQ},

Table 1. Summary of Cross Peaks Observed in the 2D HYSCORE Spectra of All Protein Solutions Investigated[a]

	Az(pae)			Az(asp)		AO
	pH 3.9 ($g_\perp$)	pH 4.8 ($g_\perp$)	pH 7.0 ($g_\perp$)	pH 4.8 ($g_\perp$)	pH 4.8 ($g_\parallel$)	pH 7.0 ($g_\perp$)
$\nu^h_{0,-}$; ν^h_{DQ}	0.8; 3.8	0.8; 3.9	0.8; 3.9	0.8; 3.8	0.9; 3.5	0.7; 3.8
ν^h_+; ν^h_{DQ}	1.5; 3.9	1.6; 4.0	1.5; 3.9	1.5; 3.9		1.5; 3.7
ν^h_+; $\nu^h_{SQ_1}$					1.5; 0.8	
$\nu^h_{0,-}$; $\nu^h_{SQ_2}$					0.9; 2.7	
ν^x_0; ν^x_{DQ}					0.6; 3.7	
ν^x_-; ν^x_{DQ}	2.0; 3.9	1.9; 4.0				
ν^x_+; ν^x_{DQ}	2.8; 3.9	2.9; 4.1	2.8; 4.0	2.8; 3.9		
ν^x_0; $\nu^x_{SQ_1}$					0.6; 2.3	0.8; 3.15[b]
ν^x_0; $\nu^x_{SQ_2}$					0.6; 1.4	

[a] The frequencies are given in MHz and the estimated error is ±0.1 MHz. [b] Tentative assignment only. See text.

3.8 *vs* 3.9 MHz (see Table 1). This shift is understood once the inhomogeneous broadening of ν_{DQ}, often showing a powder pattern like line shape,[29] and the polarization of the NQR amplitudes[20] are considered. The amplitudes of the NQR peaks are highly dependent on the orientation of the external magnetic field with respect to the principal axis system of the nuclear quadrupole tensor.[20] Therefore, the position of the cross peaks will be determined by the frequencies within the broad peak of ν_{DQ} which correspond to the orientation at which the particular NQR peak has a high intensity.

We exclude the possible assignment of the peak at (2.0; 3.9) MHz to ($\nu^{h(1)}_0 + \nu^{h(2)}_+$; ν^h_{DQ}), where the sum combination frequency $\nu^{h(1)}_0 + \nu^{h(2)}_+$ is due to the presence of two N^h,[34] on the basis of the weak modulation depth of His-46 due to its relatively low isotropic hyperfine constant ($A_{iso} = 1$ MHz).[13] This is further supported by the lack of combination peaks in the three-pulse ESEEM spectrum of type I copper in AO attributed to the relatively low value of A_{iso} of one of the remote imidazole nitrogens.[35]

The HYSCORE spectra of Az(pae) at pH values of 3.9, 4.8, 6.0, and 7.0 are similar, all showing the (ν^x_+; ν^x_{DQ}) cross peak. The details of the cross peaks appearing in each spectrum are given in Table 1. Besides the well-defined cross peaks all spectra show two ridges at (0.8; 0.8−3.3) MHz which we attribute to overlapping cross peaks involving ν_{SQ1} and ν_{SQ2} with the NQR frequencies of all three nitrogens. Unfortunately, due to the low resolution, cross peaks involving ν^x_0 were not observed.

Azurin (*Alcaligenes species*). Orientation-selective three-pulse ESEEM spectra of Az(asp) at pH 4.8 are shown in Figure 5. The peaks observed are similar to those of Az(pae) and consist of narrow, field-independent NQR signals at 0.7−0.8, 1.5, 2−2.1, and 2.8 MHz. These are assigned as in Az(pae) to $\nu^h_{0,-}$, ν^h_+, ν^x_-, and ν^x_+, respectively. The spectrum recorded at the low-field edge ($g_\parallel$ of the copper $M_I = 3/2$) exhibits a signal also at ≈0.6 MHz which is attributed to ν^x_0.[13] The double quantum frequency peaks appear in the range 4−5 MHz, and as in Az(pae) the peak is broad and structured.

The 2D HYSCORE spectrum of Az(asp), recorded at $g = 2.06$ ($\approx g_\perp$), is shown in Figure 6a. It is very similar to that of Az(pae) (see Figure 4) and the positions of the off-diagonal signals are given in Table 1. The (ν^x_-; ν^x_{DQ}) peak is weak and does not appear at the contour level at which the spectrum in Figure 4a is presented. It does become apparent, however, at

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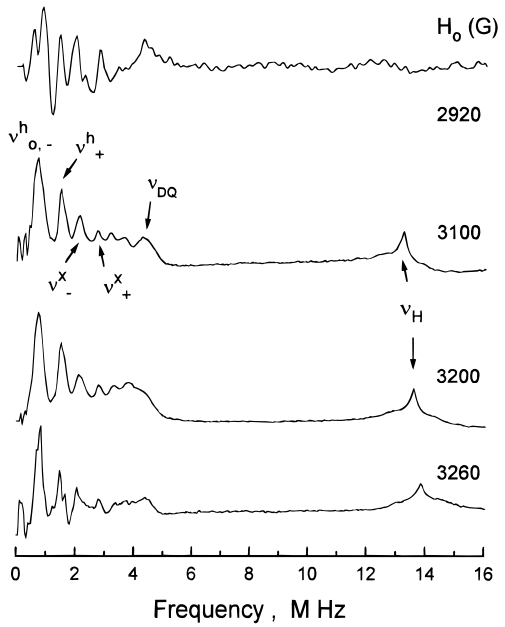

Figure 5. Orientation-selective three-pulse FT-ESEEM spectra of Az(asp) at pH = 4.8 and $\tau = 0.18$ μs. For $H_0 = 2920$ G, $\tau = 0.17$ μs.

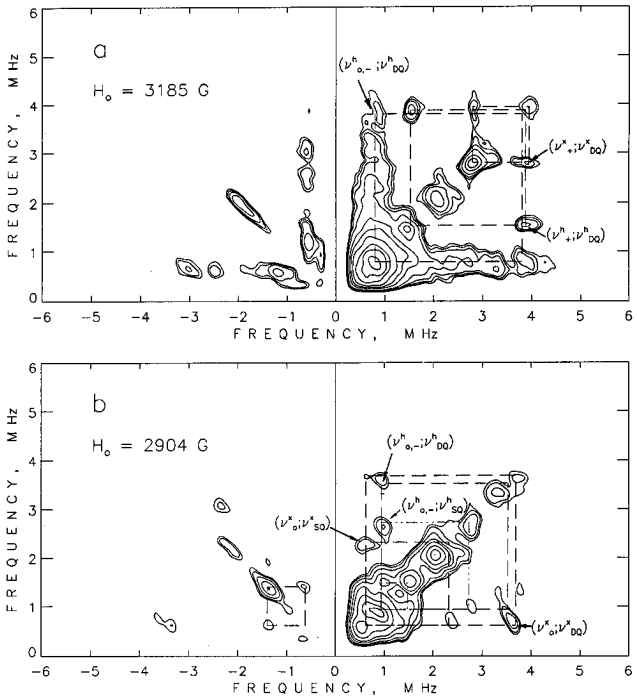

Figure 6. 2D HYSCORE spectra of Az(asp) at pH 4.8 and $\tau = 0.3$ μs: (a) $g = 2.06$; (b) $g = 2.23$.

a lower contour level. The HYSCORE spectra of Az(pae) and Az(asp) (Figure 4 and 6a) exhibit similar patterns in the (+;−) quadrant, the shape of the signal is close to that of the ridges observed in the (+;+) quadrant, though a close look reveals a slight shift to lower frequency, i.e. 0.6 MHz as compared to 0.7 MHz. We tentatively ascribe the cross peaks in the (+,−) quadrant to correlations of ν_{SQ_1} and/or ν_{SQ_2} of N^h and/or N^x with the corresponding NQR signals. A better account of the line shapes and locations of signals within the (+;+) and (+;−) quadrants requires computer simulations.

The 2D HYSCORE spectrum recorded at $g = 2.23$ ($g_{||}$ of the copper $M_I = -3/2$) is presented in Figure 6b. At this position the spectrum exhibits a better resolution since it originates from a relatively narrow range of orientations, thus generating a "single crystal" like spectrum. The cross peaks

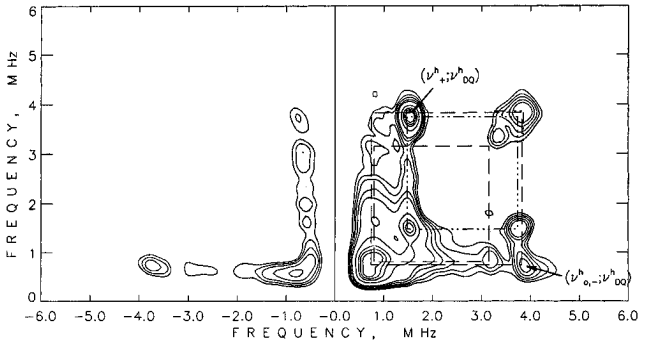

Figure 7. 2D HYSCORE spectrum of ascorbate oxidase at pH 7.0, $\tau = 0.3$ μs, $H_0 = 3200$ G, and $g = 2.06$.

observed and their assignment are listed in Table 1. This spectrum shows new signals at (0.6; 2.3), (0.6; 3.7), and (0.9; 2.7) MHz. The former two are attributed to (ν_0^x; $\nu_{SQ_1}^x$) and (ν_0^x; ν_{DQ}^x), respectively. The appearance of the third peak indicates that at this field the signal at 0.8−0.9 is a superposition of $\nu_{0,-}^h$ and $\nu_{SQ_1}^x$ or that ν_+^x and $\nu_{SQ_1}^h$ overlap at 2.8 MHz. We thus assign the (0.9; 2.7) peak to (ν_0^h; $\nu_{SQ_1}^h$) or ($\nu_{SQ_1}^x$; ν_+^x). The analysis of the peaks in the (+;−) quadrant (see below) shows that the former option is more likely.

The double quantum frequencies, ν_{DQ}^x and ν_{DQ}^h, are well resolved in this spectrum and appear at 3.8 and 3.4 MHz, respectively. Using these values and those of the single quantum frequencies, determined from the cross peaks appearing in the (+;−) quadrant, (0.6; 1.4) MHz (ν_0^x; $\nu_{SQ_2}^x$), and (0.8; 1.5) MHz ($\nu_{SQ_2}^h$; ν_+^h) in the (+;+) quadrant, the frequency of the second single quantum signal can be obtained for both N^h and N^x using the relation $\nu_{DQ} = \nu_{SQ_1} + \nu_{SQ_2}$. This yields $\nu_{SQ_1}^x = 2.4$ MHz and $\nu_{SQ_1}^h = 2.8$ MHz which is in good agreement with the assignment of the cross peaks in the (+;+) quandrant and attributes the (0.9; 2.7) MHz peak to (ν_0^h; $\nu_{SQ_1}^h$). Two-dimensional HYSCORE spectra of Az(pae) were also recorded at $g = 2.23$; the spectra, however, were not as well resolved as that of Az(asp).

Ascorbate Oxidase. The HYSCORE spectrum of AO, recorded at $g = 2.06$, is shown in Figure 7. The cross peaks observed and their assignment are summarized in Table 1. The main difference between this 2D pattern and those of Az(pae) (Figure 4) and Az(asp) (Figure 6) is the absence of the diagonal and cross peaks of N^x in the spectrum of AO even at very low contour levels. At this field, the signals from type I and type II copper sites in AO overlap,[36] but the contribution of type I is larger.[35] A previous orientation selective ESEEM study of AO showed that the double quantum frequencies of the N^hs in the type I site are different, *ca*. 3.2 and 3.6 MHz at $g = 2.06$, due to their different isotropic hyperfine coupling.[35] Moreover, ν_{DQ} of the remote nitrogens in the imidazole ligands of the type II Cu(II) is broader and appears at a somewhat higher frequency, *ca*. 4 MHz, at this g value.[35,37] The assignment of the (0.7; 3.0) MHz cross peak is rather ambiguous. It can either be the ($\nu_{0,-}^h$; ν_{DQ}^h) of the nitrogen with the lower A_{iso} or part of the ridge at (0.7; 0.5−3.0) MHz, appearing in both quadrants and representing the correlations of the single quantum transitions with the overlapping ν_0^h and ν_-^h.

Discussion

The HYSCORE spectra of native Az(pae) at pH values in the range 3.9−7.0 and that of Az(asp) at a pH of 4.8 (Figure 6)

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show the ν_+^x signal of N^x and its clear correlation with the ν_{DQ}^x peak which in the ESEEM spectrum (Figure 5) overlaps with ν_{DQ}^h of the imidazole nitrogens. This correlation provides experimental evidence for the existence of a third, weakly coupled nitrogen in native azurins from *Pseudomonas aeruginosa* and *Alcaligenes species* and confirms the previous assignment[13] of its ESEEM frequencies to the NQR and the double quantum transition. Moreover, HYSCORE measurements at the low field edge of the EPR spectrum, where a "single crystal" like spectrum is obtained, also revealed the single quantum frequencies of the coupled nitrogens, in particular $\nu_{SQ_1}^x$ and $\nu_{SQ_2}^x$. This is important since the observation of these frequencies is most helpful for the determination of the orientation of the quadrupole and hyperfine tensors of the nitrogen by computer simulations[16] and thus providing more experimental data that will lead to its unambiguous identification. The similar patterns obtained at all pH values are in agreement with earlier studies showing that the conformational changes taking place in Az(pae) do not affect the copper site.[5,38,39]

Based on the quadrupole coupling constant, 3.1 MHz, and the asymmetry parameter, 0.45, of N^x, Coremans *et al.*[13] assigned this nitrogen to an amide backbone nitrogen and suggested two possible candidates: one is the backbone nitrogen of Cys-112 where the nitrogen spin density comes through the cysteine sulfur. This backbone nitrogen is located three bonds away from the sulfur and all three bonds are aliphatic. The second possibility is the backbone nitrogen of His-46, where the spin density originates from the coordination of the copper to the Gly-45 carbonyl which is two bonds away from the nitrogen. In this case conjugation involving the nitrogen lone pair and the coordinated carbonyl is possible. Two other amide nitrogens in the vicinity of Cu(II) are the NH's of Asp-47 and Phe-114 which are hydrogen bonded to $S^\gamma-$Cys-112.[5,40] Although these hydrogen bonds may play a role in determining the spectral properties of the Cu site, it is unlikely that they provide the pathway for spin density on the amide nitrogens of Asp-47 or Phe-114.

On the basis of the comparison of the HYSCORE results of the azurins and AO we find the possibility that N^x is the His-46 backbone nitrogen more plausible. The 3D structure of the type I site in AO's is shown in Figure 1b.[6] It shows no evidence for a fifth ligand and the HYSCORE and ESEEM[35,37] spectra lack any signals corresponding to a nitrogen besides the remote nitrogens of the imidazoles. The copper hyperfine values of type I Cu(II) in Az(pae) and AO are very close ($A_{||} = 58 \times 10^{-4}$ and 59×10^{-4} cm^{-1}, respectively)[36,41] and so are the hyperfine couplings of the remote nitrogens of the imidazole.[13,35] This suggests that the spin density on the cysteine sulfur and the other nuclei of this residue should be comparable in azurin and AO. Therefore, if N^x were the cysteine backbone nitrogen, one would expect observable modulations from the backbone cysteine nitrogen also in the AO spectra. The absence of signals favors the possibility that the third nitrogen is the His-46 backbone nitrogen, connecting Gly-45. This assignment further implies that also in azurin from *Alcaligenes species*, the precise 3D structure of which is not yet available, the copper in the type I site has five ligands rather than four. The assignment of N^x to the His-46 backbone nitrogen, however, does not agree with the self consistent field Xα-scattered wave calculation which showed that interaction of an axial carbonyl oxygen of a glycine residue with the blue copper site is not covalent but rather weakly ionic.[42] The latter cannot lead to finite spin density on the corresponding amide nitrogen. The calculations were made on a model where the histidines, cysteine, methionine, and glycine ligands were replaced with ammonia, methyl thiolate, dimethyl sulfate, and formaldehyde, respectively. The different ligands may be the reason for the disagreement. Furthermore, we note that the hyperfine coupling of N^x is very small, which in turn may be a consequence of a very small degree of covalency, lower than the sensitivity of the calculations.

Conclusions

This study demonstrates that the 2D HYSCORE experiment is most useful for detecting, unraveling, and assigning ESEEM frequencies in metalloproteins. The existence of a third weakly coupled nitrogen in the type I Cu(II) site of native azurin from *Pseudomonas aeruginosa* and *Alcaligenes species* is evident from the 2D HYSCORE spectra. Comparison with the corresponding spectrum of AO suggests that this nitrogen is the amide nitrogen of His-46, magnetically coupled to the copper *via* the carbonyl group of Gly-45. This further implies that also in azurin from *Alcaligenes species*, the copper in the type I site has five ligands rather than four.

Acknowledgment. This work has been funded by the German-Israeli Foundation for Scientific Research and Development (Grant No. I-320-211.05/93) and by the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities. We thank Dr. E. Groenen from the University of Lieden for making ref 13 available to us prior to its publication and Dr. J. J. Shane from the Department of Chemical Physics, Weizmann Institute of Science for his help in the treatment of the HYSCORE data.

JA952704S

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