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**Magnetic circular dichroism anisotropy of the CuA centre of nitrous oxide reductase from coherent Raman detected electron spin resonance spectroscopy**

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Keywords:	Magnetic circular dichroism, CuA, Coherent Raman, Electron spin resonance



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5 **Magnetic circular dichroism anisotropy of the Cu<sub>A</sub> centre**  
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8 **of nitrous oxide reductase from coherent Raman detected**  
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11 **electron spin resonance spectroscopy**  
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30 Coherent Raman detected electron spin resonance spectroscopy is a technique that bridges  
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32 the established fields of magnetic resonance and magneto-optics. By exploiting the  
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34 orientational selectivity of the microwave resonance condition it becomes possible to  
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36 measure the relative orientations of the magnetic and optical anisotropies of paramagnetic  
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38 chromophores, and thereby to test models of their electronic structure. Here we report the  
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40 application of this method to the Cu<sub>A</sub> centre from *Paracoccus pantotrophus* nitrous oxide  
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42 reductase, an unusual mixed valence copper, Cu(I)/Cu(II), dimer centre also found in some  
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44 heme-copper terminal oxidases. Data from the principal visible bands (at 476, 514 and 750  
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46 nm) shows that their magnetic circular dichroism is almost entirely aligned with the g-value  
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z-axis. This is consistent with previous models of the electronic structure in which the optical transitions are polarised within the copper-thiolate plane of the centre, and the g-value z-axis is orientated normal to this plane.

*Keywords: Magnetic circular dichroism; CuA; Coherent Raman; Electron spin resonance*

## 1. Introduction

Nitrous oxide reductase (N<sub>2</sub>OR) catalyses the final step of bacterial dinitrification, the reduction of nitrous oxide (N<sub>2</sub>O) to dinitrogen (N<sub>2</sub>) [1]. It consists of two copper-containing clusters, one called Cu<sub>Z</sub> being the catalytic centre at which nitrous oxide is reduced to nitrogen, comprising a tetranuclear copper cluster bridged by an inorganic sulphide ion. The other centre, called Cu<sub>A</sub>, facilitates electron transfer to the catalytic site in N<sub>2</sub>OR and other complex enzymes such as cytochrome c oxidase (the terminal oxidase of respiration in higher organisms) and a recently identified nitric oxide reductase [2]. The centre contains two copper ions bridged by the thiolate sulphurs of two cysteine amino acid residues, forming an approximately flat rhomboid, figure 1. Each copper ion is additionally coordinated by an imidazole nitrogen from histidine lying close to the rhomboidal plane. Each copper is also relatively weakly coordinated by a longer bond to a fourth amino acid residue (one methionine sulphur, and one backbone carbonyl oxygen in N<sub>2</sub>OR) forming a highly distorted tetrahedral coordination geometry around both coppers. The electron transfer functionality of the centre is achieved by redox cycling between a reduced diamagnetic di-cuprous state and an oxidised paramagnetic mixed valence state in which the unpaired electron is essentially equally delocalised between the two copper ions. This

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3 delocalised state was first identified from the fact that the single unpaired electron interacts  
4 almost equally with each copper nucleus. The individual nuclear spins of  $3/2$  give a  
5 characteristic combined seven-line hyperfine pattern that is often particularly well resolved  
6 in the g-parallel region of the electron spin resonance “powder” spectrum of the frozen  
7 aqueous solution.  
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11 The unusual electronic structure of  $\text{Cu}_A$  has attracted study by many spectroscopic methods

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15 [1]. Low temperature magnetic circular dichroism (MCD) studies in particular have  
16 identified the principal optical transitions in the near-UV to near-IR region [3, 4]. The

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The dominant feature is a pair of oppositely signed temperature dependent MCD bands centred  
at about 500nm ( $20,000 \text{ cm}^{-1}$ ). Such a feature, which is known as a “pseudo A-term” in the  
MCD literature, is characteristic of two transitions with mutually perpendicular linear  
polarisation and similar energy whose states have been strongly mixed by spin-orbit  
coupling. The second main feature is a negative MCD band at about 750 nm ( $13300 \text{ cm}^{-1}$ ).

In order to probe the nature of the optical transitions, and hence the electronic structure, in  
more detail we would like to measure the orientational dependence of the MCD. Such  
information is, in principle, contained within the magnetic field and temperature  
dependence of the MCD of a frozen aqueous solution, since centres with different  
orientations with respect to the applied field have different g-values and hence different  
MCD field/temperature dependences [5-7]. However, in species with relatively small g-  
value anisotropies, such as copper centres, the analysis of such MCD “saturation” or  
“magnetisation” curves leads to low accuracy since it involves deconvolution of the data  
with a continuous distribution of very similar simulation functions.

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3 A much more accurate method exploits the high spectral resolution of electron spin  
4 resonance (ESR). Since g-value anisotropy is often well resolved in an ESR experiment, we  
5 can use the microwave resonance condition to select centres with specific orientations with  
6 respect to the applied magnetic field. If we can use microwave resonance to modulate or  
7 perturb the MCD, we can perform an orientationally selective MCD experiment in which  
8 the relative orientations of the MCD and g-value anisotropies can, in principle, be deduced.  
9 Essentially the same fundamental approach is widely used in a variety of electron-nuclear  
10 spectroscopies to obtain orientationally resolved measurements.

11 An early implementation of this microwave-selected MCD idea built upon established  
12 optically detected magnetic resonance principles [8-11]. These experiments use MCD to  
13 monitor changes in the spin polarisation (population difference) of the ESR transition that  
14 occur when microwave power is absorbed. Two difficulties were encountered with this  
15 approach, however. Firstly, a variety of complex magnetic relaxation phenomena [11]  
16 (cross-relaxation and spectral diffusion) tend to remove the orientational selectivity of the  
17 experiment since these processes can change the spin polarisation of centres that are not  
18 resonant with the applied microwave field [12]. It is quite possible for “inhomogenously  
19 broadened” ESR spectra to microwave power saturate “homogenously”. Additionally, the  
20 signal magnitude in this experiment is determined by the balance between the longitudinal  
21 relaxation phenomena (spin-lattice relaxation, cross relaxation and spectral diffusion) and  
22 the microwave power absorption. Simulation of data to extract MCD anisotropy therefore  
23 requires knowledge of the anisotropy (and magnetic field dependence) of these magnetic  
24 relaxation processes. Frequently such data are not available. Unravelling the anisotropies of  
25 the magnetic relaxation and the MCD is especially difficult in proteins containing multiple

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3 paramagnetic centres, and samples with unknown quantities of minor paramagnetic species.

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5 In some early work it was simply assumed that the magnetic relaxation was isotropic and  
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7 independent of magnetic field [8, 9]. Despite these difficulties, these “longitudinally  
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9 detected” experiments did show success in the deconvolution of overlapping optical spectra  
10  
11 due to multiple chemical species. These experiments relied, in effect, on distinguishing  
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13 between the different centres by virtue of their different microwave power saturation  
14  
15 characteristics. A particularly important example, and one especially relevant here, was the  
16  
17 separation of the Cu<sub>A</sub> MCD spectrum from the intense heme spectrum of cytochrome c  
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19 oxidase [9]. This experiment was one of the first to clearly identify the principle features of  
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21 the Cu<sub>A</sub> described above, although with the benefit of hindsight some minor features may  
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23 be due to perturbation of the heme MCD via magnetic interaction (cross-relaxation) with  
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25 the Cu<sub>A</sub> rather than the Cu<sub>A</sub> itself.  
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28 The magnetic relaxation related uncertainties encountered with the longitudinally detected  
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30 experiment led to a fundamental revision of the measurement strategy [12]. It is well  
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32 known that the signal magnitude in conventional ESR spectroscopy, when integrated over  
33  
34 the spectral lineshape, is independent of the magnetic relaxation processes [13]. Interaction  
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36 with other paramagnetic centres, and other interactions with the environment, leads to  
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38 changes in the ESR lineshape only. This arises from the fact that the precessing  
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40 magnetisation perpendicular to the applied field is recorded, rather than changes in the  
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42 magnetisation along the field, or, in other words, “coherence” in the spectroscopic  
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44 transition is recorded rather than spin polarisation of the stationary eigenstates. The  
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46 equivalent experiment in the MCD context is the measurement of the sinusoidally  
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48 oscillating MCD perpendicular to the applied field. The removal of the experiment's  
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3 dependence on magnetic relaxation phenomena causes a vast simplification in the  
4 interpretation and simulation. We may, for example, assume that different paramagnetic  
5 species in the sample contribute separately and linearly to the observed spectrum. Such a  
6 basic property is taken for granted in conventional ESR. Like conventional ESR we may  
7 also accurately simulate data from knowledge of the matrix elements between the  
8 (effective) spin states, other interactions and degrees of freedom can be subsumed within  
9 phenomenological lineshape functions [14].

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The measurement of the microwave induced precession of the MCD offers unprecedented  
orientational and chemical selectivity. However, this selectivity also brings a considerable  
metrology challenge. Only a very small proportion of the chromophores present in the  
sample interact resonantly with the microwaves, but they may all contribute to the optical  
absorption. The microwave modulation of the MCD in a typical experiment is therefore  
often only a very small proportion of the total conventional MCD of the sample. Typical  
signal magnitudes in metallo-enzyme samples are a hundred times smaller than the noise  
floor of the spectropolarimeters typically used in MCD spectroscopy. The single previous  
example of an ESR measurement detected by a microwave modulated magneto-optical  
effect (on a single crystal of CdS) [15], involved modulations about five orders of  
magnitude larger than a metallo-enzyme experiment. Whereas these unusually large  
modulations can be detected by spectrally resolving the modulation side-bands using laser  
interferometry [15, 16], it is highly improbable that this approach would be feasible for  
biological samples. Instead a method that had previously been used for radiofrequency  
spectroscopy of atomic vapours [17], was extended to the microwave region – optical  
heterodyne detection. The development of efficient photodetectors with microwave

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3 frequency response bandwidths enabled this method to achieve near quantum limited  
4 detection sensitivity using a few mW of visible laser light [18]. Crucial in achieving this  
5 performance is the fact that optical heterodyne detection, unlike laser interferometry, is  
6 essentially blind to fluorescence and other “incoherent” optical backgrounds. The first  
7 instrument of this type was constructed by one of us in 1995 at the Department of Physics,  
8 ETH, Zürich. This endeavour combined equipment and expertise from both the University  
9 of East Anglia, Norwich, UK and ETH. In this special edition of Molecular Physics  
10 dedicated to the memory of Arthur Schweiger (ETH), we recall his enthusiastic and  
11 generous support of the project, without which it is unlikely that instrument could have  
12 been built. Using this prototype instrument it was possible to demonstrate the first  
13 experiment on paramagnetic transition ions, namely, Cr<sup>3+</sup> in Ruby [19].

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14 The subsequent development and refinement of the microwave modulated MCD technique  
15 centred initially on its application to metallo-proteins [14, 20, 21] (with collaborators at the  
16 University of Dortmund) and more recently on its uses in solid-state physics studies [22-  
17 24]. In some contexts it is more straightforward to think of the experiment as an optically  
18 detected ESR experiment rather than a microwave modulated MCD experiment as we have  
19 emphasised here. It is also useful to recognise that the creation of microwave frequency  
20 modulation sidebands about a central laser excitation frequency can also be formulated in  
21 terms of coherent Raman processes [25]. For these reasons we follow the convention in the  
22 physics literature and refer to the experiment as a coherent Raman detected ESR  
23 experiment.

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24 When the measurement of ESR is the principal goal of an experiment, coherent Raman  
25 detection can, like other optically detected magnetic resonance (ODMR) techniques [10, 26,

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3 [27](#), offer dramatically improved detection sensitivity over standard microwave detection.  
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5 The chemical selectivity provided by optical detection is very powerful in many  
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7 applications. Coherent Raman detection complements the better known luminescence  
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9 detected ODMR methods in that it principally targets magnetic resonance in the electronic  
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11 ground level. The simplification of theoretical interpretation and maintenance of resonant  
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13 selectivity that result from the detection of the precessing spin polarisation (magnetisation)  
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15 are also often crucially important in the ESR detection context. Finally we note that a form  
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17 of coherent Raman detected ESR experiment exists, “Raman heterodyne detected ESR”,  
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19 that is related to optical hole-burning techniques rather than the MCD related technique  
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21 described here[\[28\]](#). Its feasibility is determined by relatively unusual optical relaxation  
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23 phenomena being present, and the quantitative interpretation of the experiment requires a  
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25 detailed knowledge of these processes.  
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## 2. Theory

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31 The theory of coherent Raman detected ESR of an isolated Kramers doublet (an effective  
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33 spin  $\frac{1}{2}$  system) has been described in detail elsewhere [\[14\]](#). Only the underlying principles  
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35 will be outlined here. A useful starting point is to recognise that the optical linewidths of  
36  
37 almost all condensed phase materials, including those of the sample studied here, are  
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39 considerably larger than the microwave frequency employed in a coherent Raman detected  
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41 ESR experiment. This means that we can decouple the microwave frequency dynamics  
42  
43 from the optical frequency dynamics, and the latter need therefore not be explicitly  
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45 considered. In these circumstances, it is meaningful to talk about changes in optical  
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47 absorption and dispersion on a timescale comparable to the microwave precession period.  
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3 In addition we can note that magnetic circular dichroism has the same fundamental  
4 symmetry as a magnetic dipole – both are time-odd axial vectors. These facts mean that we  
5 can visualise the experiment as a MCD vector that precesses (and nutates) in response to an  
6 applied microwave field in the same way as the magnetic moment responds. In a simple  
7 isotropic (atomic) case [29], the MCD vector precesses about the main magnetic field. The  
8 phase of the precession relative to the oscillating microwave field is the same as that of the  
9 magnetic moment in the conventional ESR experiment, and the amplitude of the precessing  
10 MCD and the magnetic moments are proportional to each other. The two quadrature phase  
11 components of the precessing MCD are analogous to the conventional ESR absorption and  
12 dispersion signals [13]. This picture also makes it easy to understand that measurement of  
13 the precessing MCD is best achieved using a circularly polarised light beam propagating  
14 perpendicularly to the applied magnetic field.

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27 Naturally the anisotropic case is more complex. It is conveniently approached by  
28 considering the motion of the (effective or fictitious) spin vector  $\bar{S}$  and expressing the  
29 MCD in terms of this spin:  $\overline{MCD} \propto \bar{C} \cdot \bar{S}$ , where  $\bar{C}$  is a matrix relating the Cartesian  
30 components of the MCD vector,  $\overline{MCD}$ , to those of the effective spin vector. This is  
31 directly analogous to the magnetic dipole case:  $\bar{M} \propto \bar{g} \cdot \bar{S}$ , where  $\bar{g}$  is the g-matrix (or  
32 “tensor”) commonly used in ESR spectroscopy [30]. In the anisotropic case the effective  
33 spin vector  $\bar{S}$  of an individual molecule will still precess in response to the applied  
34 microwave field, but it will do so about an axis other than the main applied field. More  
35 subtly, the phase of the precession, observed in the laboratory frame, will also be affected  
36 by the magnetic anisotropy. An analytical solution of the motion of the effective spin vector

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is possible however [14], and the resulting oscillating MCD can therefore also be obtained

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in terms of the elements of the matrix  $\overline{\overline{C}}$ . The components of  $\overline{\overline{C}}$  can be related to the electric dipole matrix elements of the monitored optical transition:

$$C_{il}(A \rightarrow J) = \langle A, l | C_i(A \rightarrow J) | A, l \rangle; \quad C_i(A \rightarrow J) = \frac{1}{2i} P_A (m_j P_j m_k - m_k P_j m_j) P_A;$$

where  $l = x, y, z$  are principal axes of the g-matrix;  $i, j, k = x, y, z$  and symmetric permutations of these indices;  $|A, l\rangle$  is a state of the ground level manifold A with fictitious spin quantised along the lth g-value axis;  $C_i(A \rightarrow J)$  is an operator describing the

paramagnetic ("C-term" [31]) MCD of the absorption band between manifolds A and J,

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observed along the ith g-value axis;  $m_j$  is the electric dipole operator along the jth g-value axis; and  $P_j = \sum_l |J, l\rangle \langle J, l|$  is a projection operator into manifold J (and similarly for  $P_A$ ).

In the absence of molecular spatial symmetry, all nine elements of the matrix,  $\overline{\overline{C}}$ , will be non-zero, however in a sample containing randomly orientated molecules only the three diagonal elements are observable:  $C_{xx}$ ,  $C_{yy}$  and  $C_{zz}$ . Although contributions arise from the off-diagonal elements for individual molecules, another molecular orientation will always exist that gives rise to an equal and opposite cancelling contribution. From the form of the operator  $C_i(A \rightarrow J)$  we can see that an MCD along the ith g-value axis requires non-zero electric dipoles along both axes orthogonal to it (j and k). This arises from the need to couple to circularly polarised radiation, which has fields in both directions orthogonal to the propagation axis.

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### 3. Experimental section

The sample of nitrous oxide reductase was purified from *Paracoccus pantotrophus* under anaerobic conditions, then oxidised using ferricyanide as described in detail elsewhere [3].

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The optical absorbance, electron spin resonance and magnetic circular dichroism spectra of this form of the enzyme have also been reported elsewhere [3], and support the view that the primary paramagnetic species is the  $\text{Cu}_A$ , the catalytic  $\text{Cu}_Z$  centre being primarily in a diamagnetic oxidised form. The sample was dissolved in 10 mM Na HEPES zwitterionic buffer at pH 7.0 and mixed with an equal volume of glycerol so that an optical transparent glass was formed on freezing. The protein concentration was estimated by the BCA method to be 2.13 mM of protein dimer prior to dilution with glycerol. Chemical analysis identified 11.2 Cu and 1.5 labile sulphides per dimer.

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Coherent Raman spectra were measured with an instrument described in detail elsewhere [18, 19, 21, 22]. A visible continuous-wave laser beam passes through the sample along an axis perpendicular to the applied magnetic field provided by a split-coil superconducting magnet system. Strain-free mounted low-temperature windows allow the polarisation of the light at the sample to be controlled while allowing the temperature to be reduced to approximately 1.4 K by emersion in a superfluid liquid helium bath. A microwave frequency (approx 13.8 GHz) magnetic field is applied parallel to the propagation axis of the laser beam, and its magnitude is enhanced by surrounding the sample by a high Q resonant cavity similar to that employed in conventional ESR spectroscopy [32]. For reasons outlined in section 2, a circularly polarised laser beam will be amplitude modulated at the microwave frequency by the precessing spin in this geometry. This modulation is

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3 detected with a high-speed InGaAs photodiode and the resultant microwave frequency  
4 signal is detected using a low noise microwave receiver. This detection system achieves  
5 near shot/quantum noise limited sensitivity under the conditions of these measurements.  
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9 Since the microwave signal generated by the photodiode is phase-coherent with the  
10 sample-excitation oscillator we can measure the phase of the signal using the latter as a  
11 reference for a phase-sensitive microwave detector. The microwave detection circuit is  
12 closely analogous to the “homodyne bridge” circuits commonly employed in ESR  
13 spectroscopy [32], and it is therefore possible to measure the analogues of both the  
14 absorption and dispersion phase ESR signals. In coherent Raman detected ESR it is usually  
15 optimal to measure dispersion phase spectra at relatively high microwave irradiation levels  
16 and relatively low temperatures compared to conventional ESR. In solid-state samples the  
17 dispersion phase signal does not power saturate as easily as the absorption phase signal, and  
18 can be measured with equal sensitivity in the coherent Raman detected instrument.  
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21 Changing the polarisation of the light from left to right circularly polarised changes the sign  
22 of the microwave frequency modulation. It is therefore convenient and efficient to use a  
23 photo-elastic polarisation modulator [33] to provide a secondary 50 kHz signal modulation  
24 that can be processed using conventional DSP based lock-in amplifiers. This latter  
25 modulation performs a similar noise/drift suppression role as the magnetic field modulation  
26 commonly employed in conventional ESR spectroscopy [32]. Because we do not use field  
27 modulation we record and present the data as the absolute signal rather than its first  
28 derivative with respect to field as is common in ESR.  
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#### 4. Results and analysis

The low temperature MCD of this form of the enzyme shows dominant  $\text{Cu}_A$  features at approximately 740, 526, and 474 nm [3]. These were probed with a titanium-sapphire laser (750 nm) and two lines from an argon ion laser (514 nm, 476 nm). Dispersion phase spectra measured at these wavelengths are shown in Figure 2. Since the MCD is negative in the 750 and 476 nm regions the coherent Raman detected spectra have opposite sign to the 514 nm spectrum. It is immediately obvious that the 476 and 514 nm spectra have very similar shapes compared to the 750 nm spectrum. There was no evidence of microwave power dependent changes in the lineshape.

Quantitative analysis of the MCD anisotropy requires comparison with computer simulated lineshape functions. In order to minimise the number of free parameters we first simulate the conventional X-band (9.64 GHz) ESR spectrum, figure 3. We choose to simulate the microwave frequency susceptibility rather than the experimentally recorded first derivative with respect to magnetic field. It is the overall lineshape, rather than subtle hyperfine related effects, that is important here. Our program includes copper hyperfine but assumes that the interaction is co-axial with the g-matrix. It also includes isotropic g-strain [34] through a Gaussian distribution of g-values. The spin Hamiltonian and line broadening parameters required to fit the data are very close to those needed for other  $\text{Cu}_A$  centres [35], figure 3.

Coherent Raman spectra are calculated using the same program used to calculate the conventional ESR spectrum, figure 4. Only the microwave frequency has been adjusted to that of the coherent Raman experiment (13.68 GHz), other parameters remain unchanged

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3 from the ESR simulation. The explicit expressions for the MCD modulation that need to be  
4 integrated by the program are given in reference [14]. Unlike many conventional ESR  
5 simulation programs the dispersion phase signal is calculated as well as the absorption  
6 phase signal, and because of this it must take into account the response of the system to  
7 both positive and negative microwave frequencies [14]. In the general case a spectrum will  
8 be a linear combination of three contributions from  $C_{xx}$ ,  $C_{yy}$  and  $C_{zz}$ . However in the  
9 present sample the g-matrix is nearly axial – the  $g_x$  and  $g_y$  regions of the spectrum cannot  
10 be individually resolved. This means that the  $C_{xx}$  and  $C_{yy}$  components cannot be separated.  
11 Instead we consider their average:  $C_{\perp} = (C_{xx} + C_{yy})/2$ . Figure 4 shows the simulated  
12 spectra arising from the  $C_{zz}$  and  $C_{\perp}$  contributions. The conventional ESR spectrum (figure  
13 4c) is also provided for comparison.  
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16 The data, figure 2, are fitted by changing the relative proportion of the  $C_{zz}$  and  $C_{\perp}$  spectra.  
17 These contributions need not have the same sign, but do so in this case. The data clearly  
18 shows that for the 514 and 476 nm bands the MCD is almost entirely due to the  $C_{zz}$   
19 contribution. Residual errors between the data and the fits are remarkably small, but do  
20 result in a small amount of ambiguity. We also looked for correlations between the ESR  
21 fitting parameters and the MCD anisotropy parameters. Overall an accuracy in the ratio of  
22  $C_{zz}$  and  $C_{\perp}$  of around +/- 0.05 is conservatively estimated from this data. This is an order  
23 of magnitude better than could realistically be obtained by the analysis of MCD  
24 saturation/magnetisation curves in samples such as these [5-7]. This accuracy is well  
25 illustrated by the fact that the 750 nm spectrum has a significantly larger  $C_{\perp}$  contribution,  
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3 compared to the other two bands. Although clear in this data, a MCD  
4 saturation/magnetisation curve analysis would be unable to distinguish between the  
5 anisotropy at these three wavelengths.  
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## 9 10 5. Discussion

11  
12 The results presented here are, to a first approximation, consistent with a relatively simple  
13 model for the  $\text{Cu}_A$  centre described previously [4]. This calculation approximated the centre  
14 to an ideally symmetrical and planar  $D_{2h}$  structure. The MCD is accounted for by four  
15 dominant transitions. The 476 nm region is a  $a_g \rightarrow b_{3u}$  one electron excitation (the  $b_{3u}$  state  
16 being the highest occupied orbital of the ground level) polarised along the Cu-Cu axis. The  
17 514 nm region is due to a  $b_{1g} \rightarrow b_{3u}$  transition polarised along the orthogonal S-S axis.  
18 Since these transitions are close in energy, strong mixing by spin-orbit coupling results in a  
19 “pseudo-A term” pattern of equally intense and oppositely signed MCD bands. Since  
20 neither of the un-mixed transitions contain any transition dipole polarisation perpendicular  
21 to the plane of the rhomboid an MCD can only be generated along an axis normal to the  
22 rhomboid. The 750 nm region is accounted for by two transitions in the same model which  
23 have the same symmetries as the high energy bands just described. However in this case a  
24 symmetrical “pseudo-A term” pattern is not present. This is accounted for by the bulk of  
25 the MCD intensity arising from mixing of a low lying  $B_{2u}$  level into the  $B_{3u}$  ground level  
26 by spin-orbit coupling, rather than the mixing of the excited  $A_g$  and  $B_{1g}$  levels [4]. Despite  
27 its different origin, the MCD of these transitions can also only occur along an axis normal  
28 to the rhomboid plane since the transitions  $B_{2u} \rightarrow A_g$  and  $B_{2u} \rightarrow B_{1g}$  are polarised along  
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the S-S and Cu-Cu axes respectively. Finally, the mixing of the  $B_{2u}$  level into the  $B_{3u}$  ground level by spin-orbit coupling is also the main mechanism by which g-value anisotropy is created. The mixing of copper  $d_{x^2-y^2}$  and  $d_{xy}$  orbital character, and also sulphur  $p_x$  and  $p_y$  orbital character, by this mechanism results in an orbital enhancement of the ground level g-value normal to the rhomboid plane. Thus the g-value z-axis lies perpendicular to the rhomboid plane in this model, and, because all the optical transitions involved are polarised in the plane, the MCD will be principally normal to the plane and parallel to the g-value z-axis, as observed. The observation of small amounts of MCD in the g-value x-y plane requires some mixing of states giving rise to a transition dipole along the g-value z-axis. Further mixing by spin-orbit coupling, or the breakdown of the idealised  $D_{2h}$  symmetry would allow this. Comparison with an accurate electronic structure calculation is therefore the next phase required in this work.

## 6. Acknowledgments

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## 8. Figure captions

### *Figure 1*

Basic chemical structure of the mixed valence  $\text{Cu}_A$  centre. The relatively strong nitrogen and sulphur ligation of the copper ions from cysteine and histidine amino acid residues lie symmetrically in a plane. The weaker coordination from a methionine sulphur and a carbonyl oxygen occur from the opposite sides of this plane.

### *Figure 2*

Coherent Raman detected electron spin resonance spectra of the principal bands of the  $\text{Cu}_A$  centre measured in dispersion phase (lines). Simulations (crosses) are labelled with the ratio of the magnetic circular dichroism (MCD) parameter parallel and perpendicular to the g-value z-axis. The 476 and 750 nm spectra are of opposite sign to the 514 nm spectrum, and that conventionally used in conventional ESR, because the MCD is negative for these bands.

### *Figure 3*

Conventional X-band (9.64 GHz) electron spin resonance spectra of the  $\text{Cu}_A$  centre. Absolute absorption phase susceptibility (upper) and the experimentally recorded first-derivative with respect to magnetic field (lower) are shown. The characteristic seven-line copper hyperfine is visible in the latter. A simulation of the absolute spectrum (dotted line) used g-values of 2.18, 2.025 and 2.02 and coaxial hyperfine splittings of 40 and 22 Gauss.

### *Figure 4*

Simulated coherent Raman detected electron spin resonance spectra at the frequency (13.68 GHz) of the coherent Raman experiment. Absorption (dotted lines) and dispersion (solid lines) phase spectra are shown. Experimental spectra may be fitted using a linear combination of the two simulations shown. The  $C_{zz}$  and  $C_{\text{perp}}$  contributions result from an MCD parallel and perpendicular to the g-value z-axis. A simulation of the corresponding conventional microwave detected ESR spectra is also shown for comparison.

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