Measurement of the spin concentration of metalloprotein samples from saturation-magnetization data with particular reference to cytochrome *c* oxidase

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A protocol for obtaining high-quality saturation-magnetization data from metalloprotein samples, employing a superconducting quantum interference device (SQUID) magnetometer, has previously been reported [E. P. Day, T. A. Kent, P. A. Lindahl, E. Münck, W. H. Orme-Johnson, H. Roder and A. Roy (1987) *Biophys. J.* **52**, 837–853 and E. P. Day (1993) *Methods Enzymol.* **227**, 437–463]. Following studies of several dozen different metalloprotein derivatives, the methodology has been further refined, particularly in the area of sample preparation. The details of the sample-handling procedures now in use are described, and moreover, the critical issue of verifying that contamination by paramagnetic impurities remains insignificant is considered. Importantly, it is shown that an independent determination of the quantity of paramagnetic sample present in the magnetometer is undesirable. Much more reliable parameters concerning the ground-state magnetic properties of the system

under study are obtained if enough saturation-magnetization data are collected to enable the spin concentration to be determined during the subsequent fitting procedure. As proof of the validity of this method, the results of magnetization studies on ferricytochrome *c*, ferrocyanochrome *c* and the benzohydroxamic acid adduct of horseradish peroxidase are presented. The ability of saturation-magnetization measurements to routinely determine spin concentration to within $\pm 4\%$ of accepted values is firmly established. In addition, a saturation-magnetization study has been performed on resting and fully reduced derivatives of cytochrome *c* oxidase. These results provide an illustration of the usefulness of the technique in probing some systems which have proved difficult to study by other methods. The increased difficulties inherent in obtaining meaningful data from these cytochrome *c* oxidase and other integer spin systems are delineated.

INTRODUCTION

The information obtainable from magnetization studies of metalloproteins is often complementary to that derived from other techniques like e.p.r., magnetic c.d. and Mössbauer spectroscopies. Indeed, the concerted application of some combination of these to any given problem is generally desirable. However, there are circumstances in which magnetization measurements may provide the only reasonable avenue of investigation. These would include, for example, those situations where the site of interest is a metal centre in an integer spin state, which is only a weak chromophore, or where an iron-containing moiety cannot be enriched with ^{57}Fe . In addition, the application of magnetization measurements to the investigation of systems containing weakly coupled paramagnets is often particularly advantageous in comparison with other methods.

In two recent articles concerning saturation-magnetization studies of metalloproteins [1,2] we have been critical of the methods employed by previous workers in the field. Perhaps the most important issue of contention was the quantification procedure used. Given the inherent uncertainties involved in measuring trace metal levels in biological materials, the usual practice of independently determining the concentration of paramagnet present, typically by atomic or molecular spectrophotometry, was deemed to be a mistake. Considerably more reproducible estimates of ground-state magnetic properties are obtained if the concentration of paramagnet present is de-

termined from the magnetic data alone. To date, however, acceptance of the reliability of this protocol has had to be based on reasonable argument and indirect evidence. In this paper, unambiguous evidence of the accuracy of the new quantification procedure is presented.

Using recently developed methods of data collection and manipulation [3,4], the results of saturation-magnetization measurements on derivatives of cytochrome *c*, horseradish peroxidase and cytochrome *c* oxidase are reported. The particular samples were chosen to provide a series of data sets requiring a variable (increasing) number of free parameters in the spin Hamiltonian used to fit them successfully. Moreover, because they are all haemoproteins, the concentration of samples could routinely be checked spectrophotometrically, with improved reliability compared with other kinds of metalloprotein.

The development of appropriate sample-handling procedures is, of course, absolutely crucial to the success of any physical measurement one may wish to perform on biomolecules. Only well-defined derivatives yield unambiguous results from which firm conclusions can be drawn. However, these comments probably apply more stringently to magnetic susceptibility and saturation-magnetization studies of metalloproteins than any other kind of measurement. In fact, given that commercially available superconducting quantum interference device (SQUID) magnetometers are virtually maintenance free (apart from requiring a liquid helium fill once or twice a week) and only need calibrating perhaps once a year, nearly all the major problems

that are likely to lead to the outcome of a given experiment being difficult to interpret are associated with the sample preparation. For this reason, considerable details concerning sample handling are given in the Experimental section.

EXPERIMENTAL

Instrumentation

Magnetization data were collected between ≈ 2 K and 200 K at applied magnetic fields of up to 5.5 T, using a Quantum Design magnetic property measurement system. The sample space was modified to include a slow bleed of helium gas, controlled by a needle valve, which was introduced through the bottom of the sample support rod and passed out through the evacuation valve at the top of the sample tube. The data collection and processing methodology, together with a discussion of the requirement for deuteration of samples, has been reported elsewhere [3,4].

Electronic absorption spectra were recorded using a Varian DMS 100 spectrophotometer. E.p.r. spectra were measured with a Bruker ESR 300 spectrometer, fitted with an Oxford Instruments ESR 900 liquid-helium flow cryostat.

Reagents

Hepes (sodium salt) was purchased from Sigma. Disodium EDTA and benzohydroxamic acid were obtained from Aldrich. Sodium dithionite ($+H_2O$, 85% minimum assay) was from British Drug Houses. Deuterium oxide ($> 99\%$ 2H), oxygen-free (or reagent-grade) argon and nitric oxide from any major supplier were found to be suitable for these experiments and were used without further purification. Horse heart cytochrome *c* and horseradish peroxidase were obtained as crystalline preparations from Sigma and also used without further purification. The benzohydroxamic acid-derivative of horseradish peroxidase was prepared by the addition of a 5-fold excess of benzohydroxamic acid to an approximately 0.120 mM solution of horseradish peroxidase. Formation of the required adduct was verified by electronic and e.p.r. spectroscopies [5]. Cytochrome *c* oxidase was prepared from fresh beef heart according to the method of Yonetina [6].

Sample preparation

There are four major categories of paramagnetic contaminant that must be minimized: (1) ferromagnetic impurities in the sample-holder material; (2) nuclear spin $I_N = 1/2$ nuclei (i.e. protons) in the sample and control; (3) molecular oxygen in the sample and control; (4) adventitious transition-metal ions, principally ferrous and ferric species, in the sample. These potential contaminants can be overcome as described below.

Sample holders

The sample holders are cylindrical buckets of approximate dimensions: 8 mm outer diameter by 7.5 mm in height and 0.5 mm wall thickness. Two small holes are drilled opposite each other as close to the open end of the bucket as possible, thus providing the points of attachment for the silk threads used to suspend the bucket from the sample rod. Two materials are suitable for use in the manufacture of sample holders: 'pyrolytic' boron nitride (obtainable from Union Carbide) and 'Suprasil' quartz (Heraeus Amersil). Boron nitride holders are necessary if one intends to record Mössbauer spectra in addition to magnetization data on the same sample [7]. Usually, the con-

siderably cheaper quartz is the material of choice. Despite the use of non-magnetic tooling during manufacture, we find that the surfaces of both of these materials become seriously contaminated with paramagnetic impurities. We have determined empirically that removal of at least 2% of the initial mass of each holder by etching for several hours in 5% (w/w) hydrofluoric acid solution significantly reduces the level of contamination. After rinsing with distilled water and acetone, the etched holders are handled only with plastic-coated tweezers during filling and subsequent loading into the magnetometer.

Spin $I_N = 1/2$ nuclei

If the metalloprotein in question cannot be obtained in pelleted form, which may then simply be dissolved in deuterated buffer, it is most convenient to prepare deuterated samples in an Amicon ultrafiltration device. First, the sample volume is reduced to obtain the required concentration (usually the more concentrated the better). Next, the sample is diluted 5-fold by the addition of the appropriate deuterated buffer, then reconcentrated. The sample is then again diluted 5-fold in deuterated buffer and finally reduced in volume once more to the experimentally required concentration. The filtrate from the second buffer-exchange step is retained for use as the control. In the case of metalloproteins that are denatured by mechanical stirring, we have found the Amicon 'Centriflo' system of greater use than the more usually employed 'Diaflo' type of apparatus. To date, it has never been necessary to seek alternative procedures.

The use of very pure deuterated reagents to make up buffers is unwarranted. A good improvement in the sensitivity of magnetization measurements can be gained by achieving $\approx 95\%$ deuteration of exchangeable protons. Doing any better than this requires significantly more effort and leads to minimal additional improvement in sensitivity. The undeuterated salts of sulphonic acid-type buffers (e.g. Hepes) when dissolved in deuterium oxide may safely be used at concentrations of up to 100 mM without introducing significant numbers of slow-relaxing protons.

Molecular oxygen

Any rudimentary degassing manifold connected to a single-stage rotary pump and a supply of argon at a pressure of slightly over 1 atm (1 atm = 101325 Pa) will suffice for removal of oxygen from samples and controls. Two glass tubes must be independently connected to the manifold, one flat-bottomed and containing the sample holder to be filled, the other pear-shaped at the lower end and containing the solution to be degassed. These need to be at least 4 in (10 cm) deep and closed at the top with a gas-tight, but easily removed, stopper such as a lightly greased rubber bung. The opening at the top must be large enough to allow the insertion of a pipettor for sample transfer and forceps for removal of the loaded holder. It is recommended that the joints between the two glass tubes and the manifold be of the ground-glass ball-and-socket variety, secured with a clamp.

The degassing procedure starts by evacuating the tube containing the sample holder and flushing with argon several times, finally leaving the container under low positive argon pressure. Next, the solution in the other tube is degassed in standard fashion by two cycles of freezing-evacuation-thawing and then is also left under low positive argon pressure. The stopcocks to both tubes are now opened to the argon line and the gas pressure increased, so that when the stoppers are removed there is a steady flow of argon from the top of each tube, preventing entry of air. Following removal of the stoppers, a pipettor of the 'positive displacement' type (i.e. with plunger) is held with its tip in the

argon flow and is pumped a few times to flush out any residual air. The required volume of solution is then withdrawn and the pipettor tip moved quickly to the tube containing the sample holder. After delivery of the solution to the sample holder, the pipettor is removed, the stopper replaced in the top of the tube, the stopcock to the argon line closed and the sample (or control) frozen by immersion in liquid nitrogen, for storage and subsequent transfer to the magnetometer. At this point, it is recommended that another aliquot of the solution be withdrawn by means of a syringe fitted with a Teflon 'needle' (Hamilton) and transferred to an e.p.r. tube to check for paramagnetic impurities.

Note that the volume of the solution being degassed will normally be < 0.5 ml, while the tube in which the procedure is carried out probably has a volume in excess of 50 ml. Consequently, two cycles of freezing–evacuation–thawing should easily reduce the oxygen content of the solution to < 0.1 % of its initial value. We have occasionally used gentler methods of degassing, such as that suggested by Petersson and Ehrenberg [8]. However, while alternative methods may certainly work, the visual indication of successful deoxygenation afforded by the appearance of bubbles in thawing solutions is so useful that, in practice, this renders other procedures less than desirable.

The major difficulty with our method of choice is that freezing can denature some proteins. In fact, this is less of a problem in the case of very concentrated protein solutions and can invariably be overcome entirely by the addition of cryoprotectants like ethanediol and glycerol, both of which can be purchased in fully deuterated form (Cambridge Isotope Laboratories). It is recommended that the activity of metalloprotein samples be measured before and after degassing to check for denaturation. A reviewer has enquired as to whether the appearance of some adventitious metal ions in samples could be due to protein denaturation. This is certainly possible if metalloproteins are handled inappropriately. However, the major paramagnetic contaminant, iron (see below), is clearly scavenged from the environment since it also becomes concentrated in samples of metalloproteins like manganese superoxide dismutase [1] and urease [2], neither of which contains iron as a cofactor.

It should also be noted that syringes, particularly those fitted with stainless-steel needles, are to be avoided in the preparation of samples. They do not deliver accurate volumes of viscous solutions and more seriously, the steel needles most often employed can lead to significant contamination of protein samples with adventitious paramagnetic metal ions. Any small volume pipettor of the positive displacement type is probably suitable for use in these preparations. We presently employ Digital Varimetric Micropipettors (Labindustries).

Adventitious metal ions

High-spin ferric ($S = 5/2$) and ferrous ($S = 2$) species are the most important. If these can be minimized, then other potential paramagnetic metal-ion contaminants will probably have been taken care of. In addition to general cleanliness, there are two important considerations in maintaining low levels of adventitious iron species. First, minimize sample handling and do not let biological preparations come into contact with metal equipment such as stainless-steel fermentation vessels, spatulas and syringe needles. Secondly, maintain approximately 2 mM levels of EDTA in all buffers employed at each stage of any given preparation, especially the deuterated buffers used in the final exchange and concentration steps. It is our experience that the most serious contamination occurs in the final stages of sample preparation when the protein has been concentrated. Consequently, alternative procedures, such as those involving the use

of chelex treatments in the earlier stages of preparation, have not proven particularly advantageous, merely unnecessarily complicated.

Despite taking all reasonable precautions, it is never possible to guarantee the absence of adventitious iron from a sample. Consequently, it is always advantageous to prepare e.p.r. samples in tandem with SQUID magnetization samples as described above, in order to check for the possible presence of ferric and ferrous species. It is important to realize that the chelating properties of the protein are of the utmost significance here. In general, contaminant levels can be expected to be an order of magnitude larger in concentrated protein solutions than in control (i.e. buffer) solutions if, for instance, both are transferred with the same syringe and stainless-steel needle. Also, note that increased degrees of contamination are often experienced when dealing with reduced samples, particularly where sodium dithionite has been used as the reductant.

Data fitting

The spin Hamiltonian used to calculate the saturation-magnetization curves shown in the Figures is

$$H = D[S_z^2 - S(S+1)/3 + (E/D)(S_x^2 - S_y^2)] + \beta \sum_{i=x,y,z} g_i S_i H_i$$

where g_i , S_i and H_i are the x , y and z components of the g -tensor, the electron spin operator and the applied magnetic field, respectively; D and E/D are the zero-field splitting parameters. The x,y,z coordinate frame was fixed relative to the molecule. Unless stated explicitly to the contrary, fits reported herein are unique. Further details of the fitting procedure are described elsewhere [3,4]. The software used is now commercially available (WEB Research Co., Edina, MN, U.S.A.).

RESULTS

The saturation-magnetization data for ferricytochrome c are given in Figure 1. Measurements were made at applied magnetic fields of 5.0, 2.5, 1.25 and 0.625 T, but only the data at 5.0 and

Figure 1 Magnetization of horse heart cytochrome c samples in the temperature range 200–2 K

Ferricytochrome c at applied magnetic fields of 5.0 (○) and 2.5 (△) T, and (●) ferrocyclochrome c at 5.0 T applied field. Nominally 128 nmol protein: 150 μl of 853 μM haem c (determined spectrophotometrically) in 25 mM Hepes 2 mM in EDTA, pH 7.2. The solid line represents a best fit to the data calculated for 128 nmol of spin $S = 1/2$, with $g_{z,y,x} = 3.06, 2.24, 1.24$.

Figure 2 Magnetization of the benzohydroxamic acid adduct of horseradish peroxidase in the temperature range 200–2 K at applied magnetic fields of 5.5 (○), 2.75 (△) and 1.375 (□) T

Main figure: magnetization; inset: susceptibility. Nominally 14.8 nmol protein: 150 μ l of 99 μ M protohaem (determined spectrophotometrically) in 25 mM Hepes 2 mM in EDTA, p^H 7.2. The solid line represents a best fit to the data calculated for 14.2 nmol of spin $S = 5/2$, with $g_{x,y,z} = 1.99, 1.925, 1.925$, $D = 14.4$ cm^{-1} and $E/D = 0.014$.

2.5 T are shown, since all lie on the same curve. The filled circles represent a 5.0 T data set obtained for the sodium dithionite-reduced sample, which show ferrocycytochrome *c* to be diamagnetic ($S = 0$) as expected. The concentration of the oxidized sample was determined to be 853 μ M using $\epsilon_{410} = 106$ $\mu\text{M}^{-1} \text{cm}^{-1}$ after Margoliash and Frohwirt [9] and the volume was 150 μ l. The solid line in Figure 1 is a best fit to all four sets of data collected using $g_{x,y,z} = 3.06, 2.24, 1.24$ [10] and represents 128 nmol of an $S = 1/2$ system, in perfect agreement with the quantity of sample loaded into the magnetometer (853 μ M $\times$ 150 μ l = 128 nmol).

Note that throughout this paper we have chosen to represent raw data on a vertical axis in units of magnetization (M) per sample (rather than per mole). This is to emphasize the fact that the fit determines both the spin state and spin quantification. It should be pointed out at this juncture that horse heart cytochrome *c* is the only metalloprotein for which we have obtained such good agreement between the quantification determined by the saturation-magnetization data and an independently measured metal content. Most of the relevant extinction coefficients of other metalloproteins are not known with great accuracy and alternative methods of quantification do not offer any significant improvement. Normally we are satisfied if the metal contents of a given sample determined from the saturation-magnetization data and in some other independent manner agree to within 8% of each other [1].

Saturation-magnetization data for the benzohydroxamic acid-adduct of horseradish peroxidase at applied magnetic fields of 5.5, 2.75 and 1.375 T are shown in Figure 2. The same data are presented as magnetic susceptibility (i.e. magnetization divided by applied field) in the inset in order to show more clearly the quality of the fit obtained for all fields simultaneously. In this case, the data collected at different applied magnetic fields are not superimposable, indicating a paramagnetic ground-state of spin of $S > 1/2$. The concentration of this sample was determined as the pyridine haemochromogen using $\epsilon_{557} =$

Figure 3 Magnetization of resting bovine cytochrome *c* oxidase in the temperature range 200–2 K at applied magnetic fields of 5.5 (○), 2.75 (△), 1.375 (□) and 0.2 (◇) T

Main figure: magnetization; inset: susceptibility. Nominally 56 nmol protein: 160 ml of 700 μ M total haem *a* (determined spectrophotometrically) in 20 mM Hepes, 1 mM in EDTA, 1% Tween 80, p^H 7.8. The appropriate amounts of spin $S = 1/2$ contributions from haem *a* and Cu_x have been subtracted from the data, as has the contribution from the $S = 5/2$ adventitious ferric iron contaminant. The solid lines are best fit to the data calculated for 60 nmol of spin $S = 2$, with $g_{av} = 2.1$, $D = -8.0$ cm^{-1} and $E/D = 0.28$.

35 $\text{mM}^{-1} \text{cm}^{-1}$ after Paul et al. [11]. The concentration was found to be 99 μ M and the volume was 150 μ l. The solid lines in Figure 2 are the best fit to the data using $g_{x,y,z} = 1.925, 1.925, 1.99$ and $E/D = 0.014$ [5]. The fit suggests that the data represent 14.2 nmol of an $S = 5/2$ system with $D = 14.4$ cm^{-1} . This value for the zero-field splitting parameter is in excellent agreement with that reported (≈ 15 cm^{-1}) by Schulz et al. [5], but the quantification is 4% lower than that determined spectrophotometrically (99 μ M $\times$ 150 μ l = 14.8 nmol).

The extinction coefficient for the pyridine haemochromogen of iron-protoporphyrin IX has been determined independently to three significant figure precision by several groups [11] and is almost certainly reliable. Ultimately, the accuracy of a spin quantification determined magnetometrically depends on the absolute error on the magnetic standard used to calibrate the SQUID. In practice, this means that any relative difference between a spin quantification measured by analysis of saturation-magnetization data and the accepted (or otherwise determined) value of $< 2\%$ is insignificant. The fact that the difference obtained for the benzohydroxamic acid-adduct of horseradish peroxidase was greater than this is not surprising however. The present sample was deliberately prepared dilute to demonstrate how well the saturation-magnetization data establishes the zero-field splitting parameter D , even when the technique is pushed beyond the limits of sensitivity within which we usually try to work. So despite using less than one-third the amount of sample we would normally employ for an $S = 5/2$ system, the discrepancy between the spectrophotometrically determined haem content and that found in the fit to the magnetization data is well within our acceptable range of $\leq 8\%$.

In Figure 3 are shown saturation magnetization data for resting (i.e. as isolated) cytochrome *c* oxidase at applied magnetic fields of 5.5, 2.75, 1.375 and 0.2 T. Again, the same data are

presented as magnetic susceptibility (i.e. magnetization divided by applied field) in the inset in order to show more clearly the quality of the fit obtained for all fields simultaneously. The contributions to the measured magnetization from haem a ($S = 1/2$) and Cu_A ($S = 1/2$) have been subtracted from the data of Figure 3 using the value $\Delta\epsilon_{604} = 11 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the differential extinction coefficient (reduced minus resting) [6] to determine the total haem concentration. The remaining contribution from the haem a_3 - Cu_B site represents about 60 % of the total signal magnitude of the raw data. In addition, a contribution of 8 % (relative to total haem) adventitious ferric iron, determined by double integration of the $g = 4.3$ e.p.r. signal at 20 K and comparison with a ferric-EDTA standard, has been subtracted. Therefore, the data of Figure 3 represent the saturation-magnetization/susceptibility of the haem a_3 - Cu_B oxygen-binding site. The solid lines are the best fit to the data where D , E/D , g_{av} and the concentration are all free parameters; we find: $D = -8.0 \text{ cm}^{-1}$, $E/D = 0.28$ and $g_{\text{av}} = 2.1$ for 60 nmol of $S = 2$. There is no evidence in these data for uncoupling below 200 K of the haem a_3 ($S = 5/2$) and Cu_B ($S = 1/2$), which are therefore strongly antiferromagnetically coupled to produce a spin $S = 2$ ground state, in agreement with previous magnetic susceptibility studies [12,13]. The quantity of 60 nmol determined by the magnetization measurements is 7 % higher than the 56 nmol ($160 \mu\text{l} \times 350 \mu\text{M}$) estimated spectrophotometrically. Again, this is within the normally acceptable error range.

Saturation-magnetization data at four applied magnetic fields between 5.5 and 0.2 T (not shown) were also collected for reduced (excess sodium dithionite) cytochrome c oxidase. In this derivative, haem a , Cu_A and Cu_B were assumed to be diamagnetic. Only a contribution of 8 % (relative to total haem) adventitious iron, now assumed to be high-spin ferrous ($S = 2$) was subtracted from the raw data, which were then taken to represent the saturation-magnetization/susceptibility of reduced haem a_3 . As before, with D , E/D , g_{av} and the spin concentration all free parameters in the fit, we found: $D = 8.6 \text{ cm}^{-1}$, $E/D = 0.24$ and $g_{\text{av}} = 2.1$ for 50 nmol of $S = 2$. In this case, the 50 nmol determined from the magnetization measurements is 11 % lower than the 56 nmol estimated spectrophotometrically, 3 % outside our usual error range. However, using the alternative differential extinction coefficient (reduced minus resting) $\Delta\epsilon_{604} = 12 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ recommended by Van Gelder [14], we recalculate the amount of paramagnet present to be 51 nmol, which is in very good agreement with the magnetization data.

Oxidized samples of cytochrome c oxidase always contain adventitious copper ($S = 1/2$) that we cannot quantify with certainty. Therefore, the spin quantification for the resting derivative is likely to be systematically high. Unfortunately, the spin quantification for the reduced derivative is likely to be systematically low for the following reason. We have observed with other samples, that prolonged (i.e. 30–40 min) reduction of cytochrome c oxidase with sodium dithionite results in up to 40 % of haem a_3 being in a diamagnetic form; that is to say, the magnitude of the magnetization obtained appears to decrease with increasing reduction time. Since, to ensure full reduction of cytochrome c oxidase, it is typically necessary to allow at least 10 min to elapse following the addition of sodium dithionite, it is not surprising that some conversion of haem a_3 to the diamagnetic form should take place. Given these practical difficulties: (1) the magnetization data confirm that the differential extinction coefficient for cytochrome c oxidase at 604 nm is probably in the reported range 11 – $12 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (total haem) but cannot distinguish between these possibilities; (2) the agreement between the magnetization- and spectrophotometrically-measured haem a_3 contents of the derivatives, considering all the data together,

is outside the normally acceptable range of $\pm 4\%$ for entirely understandable reasons and therefore should not be rejected.

DISCUSSION

Quantification of spin concentration of magnetization data

In the past, most 'magnetic susceptibility' studies on metalloproteins have been performed at a single applied magnetic field and over a limited temperature range, typically where the Curie law was obeyed. In such experiments, it is absolutely necessary to obtain an independent determination of the concentration of the paramagnet under investigation before information concerning its ground-state magnetic properties can be extracted. If, however, data is collected at multiple applied magnetic fields, over a sufficiently wide temperature range to include the saturation-magnetization region as previously suggested [3,4], then the spin concentration is determined by these data. In fact, it actually turns out to be undesirable to use an independently determined spin concentration in fitting saturation-magnetization measurements because a small error in the estimation of the amount of paramagnet present can lead to quite erroneous values being found for its spin Hamiltonian parameters. For example, imposing a concentration error of 5–10 % on a fit to data like that of Figure 2 leads to an estimate of D which is incorrect by a factor of 2–3.

A number of protein derivatives have now been studied where the quantity of paramagnet present was a free parameter during the fitting procedure. These examples, presented in Table 1, show good agreement between the sample spin concentrations measured spectrophotometrically and those determined from the magnetization data. On two occasions, the first involving a reduced ferredoxin II sample and the second a reduced superoxide dismutase sample, we found 15–20 % more paramagnet present according to the fits to magnetization data than were estimated to be present spectrophotometrically. Subsequently, this was demonstrated to be due to contaminating high-spin ferrous species. In conclusion, provided the possible contaminants are accounted for (i.e. shown by e.p.r. to be negligible) our quantifications are always in agreement with independent determinations. In fact, spin quantifications are actually more reliably performed by these magnetization procedures than by other methods in a significant number of cases (e.g. [1,2,15,16]).

Quantification of adventitious iron by e.p.r.

In the case of air-stable derivatives, the problem is to quantify the high-spin ferric signal appearing at $g = 4.3$. This arises from the middle Kramers' doublet of the $S = 5/2$ ground state [17] and might therefore be expected to exhibit considerable variation in intensity between samples with different zero-field splitting parameters, leading to difficulty in selecting suitable integration standards. In fact, this turns out to be a much less serious problem than one might anticipate. If one quantifies the $g = 4.3$ signal in a protein sample by double integration of the x -band e.p.r. spectrum between 120 and 180 mT, using ferric-EDTA as the standard, then there is generally only a small change in the result ($< 20\%$) if the measurements are made at 20 K rather than 10 K. This observation seems to suggest that the environments of ferric-iron bound adventitiously to a protein and in an EDTA complex are not grossly different so far as zero-field splitting and band shape are concerned and thus, ferric-EDTA is probably a useful integration standard. Also, in those cases where magnetization measurements have previously been made on spin $S = 1/2$ samples in the presence of small amounts of spin $S = 5/2$ contaminant, the difference in form of the magnetization

Table 1. Comparison of spin concentration determined spectrophotometrically and from saturation-magnetization data

Derivative	Spin (<i>S</i>)	Spin content (nmol)			Reference
		Spectrophotometric	SQUID	Difference (%)	
Oxidized <i>E. coli</i> sulphite reductase	5/2	36.4	37.0	1.6	15
Horse heart ferricytochrome <i>c</i>	1/2	128	128	0	This work
Benzohydroxamic acid adduct of horse-radish peroxidase	5/2	14.8	14.2	4.0	This work
<i>D. gigas</i> ferredoxin II					
Oxidized	1/2	98	104	5.8	19
Dithionite reduced	2	98	100	2.0	19
<i>T. thermophilus</i> superoxide dismutase					
Oxidized	2	49.2	47.1	4.3	1
Dithionite reduced	5/2	52.5	51.3	2.3	1
Beef heart cytochrome <i>c</i> oxidase					
Resting	2	56*	60	6.7	This work
Dithionite reduced	2	51*	50	2.0	This work

* See the comments in the Results section of the text.

Table 2. Comparison of $S = 5/2$ impurity content of nominally $S = 1/2$ samples determined by e.p.r. and from saturation-magnetization data

Derivative	$S = 5/2$ content (%)		
	e.p.r.	SQUID	Reference
Nitrite adduct of <i>E. coli</i> sulphite reductase	3.0*	2.4	15
Nitrite adduct of spinach nitrite reductase	4.5*	3.0	15
Oxidized <i>D. gigas</i> ferredoxin II	2.0†	2.0	19
Reduced porcine uteroferrin	2.0†	1.6	16

* Includes contributions from both $g = 4.3$ and $g = 6$ species.

† These numbers were not explicitly reported in the original papers.

curves obtained from these two ground states is large enough that reliable quantification of the impurity content is possible from the magnetization data alone. There are several such examples on record, where quantification of the impurity content has been found both by e.p.r. and analysis of the magnetization data. These measurements, documented in Table 2, indicate that one generally overestimates (20–30%) the impurity content by double integration of the e.p.r. spectrum as described. Consequently, provided the experimental requirement is to establish that the spin $S = 5/2$ impurity content is 'low,' i.e. less than some critical level below which it insignificantly distorts the data, then the method is reliable.

As far as reduced derivatives are concerned, it is necessary to prepare a ferrous–NO complex in order to produce an e.p.r. detectable spin $S = 3/2$ system [18]. We have not found a suitable integration standard for use in quantifying this signal. One could of course overcome this problem by the addition of a known amount of ferrous sulphate to the protein sample as an

internal standard. However, to date, we have invariably been able to use the alternative strategy of (1) improving our sample handling protocol to reduce the contaminant to a barely detectable and thus negligible level, or (2) determining the impurity concentration in the air-stable form before reduction and assuming no further contamination takes place. Should these assumptions be invalid there is usually an indication in the saturation-magnetization data (see above).

A strategy for metalloprotein studies and its limitations

It is desirable to make measurements on both an air-stable and a reduced derivative drawn from the same stock preparation. Since both the paramagnetic centres of interest and the contaminants will probably have different spin states in these two situations, agreement between the quantifications obtained for the two derivatives provides an intrinsic experimental check on the validity of the fitting procedure employed and confirmation that contaminants were not a problem. We expect to find a variation of no more than 8% in the spin concentrations of derivatives prepared from the same stock, as was the case for ferredoxin II [19] and manganese superoxide dismutase [1]. The larger variation than this obtained in the present study for cytochrome *c* oxidase is explained in the Results section.

Fortunately, many metalloproteins either contain mononuclear sites of interest, like the haem in cytochrome *c* and horse radish peroxidase (this work), or they have multinuclear sites where the metal ions are tightly coupled throughout the experimental range and so behave as mononuclear paramagnets. Ferredoxin II [19] and cytochrome *c* oxidase (this work) are examples of the latter kind. Magnetization data derived from systems of this type can be fitted with up to five free parameters in the spin Hamiltonian: spin state (S), average g value, zero-field splitting parameters D and E/D and finally the spin concentration. Often, some of these are already known from other measurements, principally e.p.r. spectroscopy. Note that if our recommended procedure of studying an air-stable and a

reduced derivative of each metalloprotein is followed, then one of the pair will normally have an e.p.r. signal associated with it.

To date, substantiated cases where derivatives of native metallobiomolecules exhibit weak magnetic coupling (i.e. have multiple paramagnetic sites that become uncoupled in the accessible temperature range) are limited to rather few examples. While these systems are certainly amenable to study (e.g. [16,20,21]) they invariably require much more effort to fit the magnetization data successfully. Moreover, additional information will probably be required if an unambiguous fit is to be achieved.

The most difficult kind of metalloprotein to study is one in which there may be more than one distinct metal centre, but all the sites appear to exist in the same e.p.r. silent, paramagnetic spin state. From the magnetization data alone, it may not be possible to distinguish coupled from uncoupled sites. The nickel(II)-containing enzyme, urease, falls into this category [2]. The key problem here is to extract as much information as possible, while resisting the temptation to overinterpret the data, as discussed in detail elsewhere [4].

Finally, it must be acknowledged that there are a number of potentially interesting metalloprotein derivatives that cannot be studied at present because of high levels of paramagnetic impurity. This remains the most important limitation of the technique. A case in point concerns cytochrome *c* oxidase. Preparations of this enzyme have been known for some time to become 'pulsed' (i.e. additionally activated) during turnover [22]. Consequently, a saturation-magnetization study of pulsed derivatives is desirable in order to determine whether the coupling of the metal centres at the oxygen-binding site has changed relative to the resting form(s). Of particular interest is a hydrogen peroxide adduct [23] that may very well closely resemble an intermediate in the catalytic cycle. Unfortunately, attempts to convert resting cytochrome *c* oxidase preparations into the peroxide adduct or into the pulsed form (involving full reduction followed by reoxidation) always seem to result in significantly increased levels of paramagnetic contaminants. As the starting enzyme preparation is likely to already contain unquantifiable amounts of such contaminants (see Results section) analysis of magnetization data obtained from this kind of subsequently prepared derivative is rendered wholly unreliable.

Magnetic properties of the cytochrome *c* oxidase derivatives

In addition to providing a good illustration of the major limitation of the technique, saturation-magnetization studies of cytochrome *c* oxidase derivatives also provide some outstanding examples of the particular usefulness of such measurements. The focus of much current attention in this field is the ligand-binding haem a_3 -Cu_B pair, which can be prepared in a number of interesting integer spin states [24]. As far as highly active preparations of the eukaryotic enzyme are concerned (and for a number of practical reasons) most of these integer spin derivatives are not readily amenable to study by e.p.r., low-temperature magnetic c.d. or Mössbauer spectroscopy. However, in principle, all can be usefully examined by saturation-magnetization measurements.

Consider, for example, the problem of the variation in functional characteristics of fully oxidized cytochrome *c* oxidase prepared by different methods [24,25]: are these due to structural changes at the haem a_3 -Cu_B pair, or other factors, such as differences in protein tertiary structure? Baker et al. [26] have described purification procedures for bovine cytochrome *c* oxidase which lead to preparations exhibiting either 'fast' or 'slow' reaction with added cyanide in their oxidized forms. Cytochrome

c oxidase samples prepared by the Yonetani method, like those used in this study, react slowly with added cyanide in the resting (i.e. as isolated) form. A saturation-magnetization study of oxidized fast and oxidized slow cytochrome *c* oxidase has recently been published [27]. Significantly, while the ground state magnetic properties of the haem a_3 -Cu_B site in the oxidized fast enzyme were quite distinct, those of the oxidized slow enzyme were very like the properties reported here for resting cytochrome *c* oxidase. In particular, there seem to be no other substantiated cases of haemoprotein derivatives exhibiting negative axial zero-field splitting parameters (*D*) in the literature. This observation is quite important. The possibility that the observed differences between fast and slow oxidase might simply be a function of pH was not explicitly addressed in the earlier study. The present data were collected on samples prepared under mildly alkaline conditions, rather like those employed in the preparation of the fast derivative in the previous work and unlike the mildly acidic conditions used to prepare the slow derivative. Consequently, the magnetization data strongly suggest the electronic structure of the haem a_3 -Cu_B site to be the same in resting and slow cytochrome *c* oxidase, but clearly different in the case of the fast enzyme [27]. Moreover, of course, these results indicate that the observed variation in functional characteristics of cytochrome *c* oxidase preparations is at least partly due to changes intimately connected with the coordination sphere of the haem a_3 -Cu_B pair.

This research was supported by a Grant-in-Aid from The American Heart Association (Alabama Affiliate) and by Biomedical Research Support Grants S07RR07151-11 and -12 (J.P.), National Institutes of Health Grant GM32394 (E.P.D.) and awards from the Science and Engineering Research Council (M.T.W.).

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Received 6 June 1994/30 August 1994; accepted 15 September 1994