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INTENSITY OF HIGHLY ANISOTROPIC LOW-SPIN HEME EPR SIGNALS

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Summary

A semi-empirical formula has been derived to calculate the concentration of low-spin heme compounds that are highly anisotropic, i.e. $3 < g_z < 4$, and where information only on the g_z absorption is available.

Introduction

EPR can be used for the determination of the concentration of paramagnetic species through comparison with a known standard. In the procedure developed by Aasa and Vänngård [1] the total intensity of a rhombic powder spectrum is calculated from the area under an isolated absorption peak in the first derivative spectrum. This area is related to the intensity of the signal through a proportionality factor which is a function of the three g values.

Since in general only one or two g values can be detected in low-spin heme compounds with highly anisotropic EPR spectra especially if g_z is greater than 3.3, the concentration of these compounds could not be determined by EPR.

Based on the expressions of the g values of low spin $3d^5$ systems, derived by several authors [2—5], the proportionality factor of Aasa and Vänngård can be rearranged into a form, that is exclusively a function of g_z. Since it is always possible to detect the g_z peak, the concentration of low-spin heme compounds with highly anisotropic EPR spectra can now be determined by EPR.

Materials and Methods

Cytochrome *c* and the cyanide complexes of metmyoglobin and cytochrome *c* were prepared as described in the literature [6]. The concentration of cytochrome *c* was determined optically using $\Delta\epsilon^{550}_{red-ox} = 21.1\ mM^{-1} \cdot cm^{-1}$ [7] and

that of metmyoglobin according to Ref. 8. EPR measurements and digitizing of EPR spectra were performed as described in Ref. 9. EPR spectra were analyzed with a Du Pont 310 Curve Resolver.

Results

EPR theory of low-spin $3d^5$ systems

Many authors have derived expressions of the g values of low-spin heme compounds in terms of the wave-function coefficients [2—5].

Putting the orbital reduction factor k equal to 1 and $g_e = 2$, Griffith [3,10] calculated that the sum of the squares of the three g values, S, maximally equals 16. If $g_e = 2.0023$ this sum equals 16.018. For the derivation we put $g_e = 2$. From the expressions of the g values [11] it follows:

$$A = \tfrac{1}{2}\sqrt{2(\tfrac{1}{2}g_z + B^2)^{1/2}} \tag{1}$$

$$p^2 = \tfrac{1}{2}g_z + 2B^2 + B(2g_z + 4B^2)^{1/2} \tag{2}$$

where $p = \sqrt{2A + B}$. The sum of the squares of the g values, S, can then be expressed:

$$S = g_x^2 + g_y^2 + g_z^2 = 4p^2(4 - p^2) \tag{2A}$$

By a calculation of the wave-function coefficients from the experimental g values of low-spin heme compounds and from the data in Ref. 5 it appeared that $2 < C/B < 5$ (C is a wave-function coefficient).

Table I shows a calculation of S at different values of g_z and B. In those

TABLE I

CALCULATION OF THE SUM OF THE SQUARES OF THE g VALUES (S) AT DIFFERENT VALUES OF g_z AND B

From the values given in Ref. 5 it appears that usually $B < 0.2$ and $2 < C/B < 5$ in those low-spin heme compounds having a set of g values comparable with experimental data. A good estimate for A from Eqn. 1 is $A = 1/2(g_z)^{1/2}$, since B is small. The maximum for B is $B_{max} = (1-A^2)^{1/2}$, when $C = 0$. As $2 < C/B < 5$ and $A^2 + B^2 + C^2 = 1$ it follows that $0.2\,B_{max} < B < 0.45\,B_{max}$. It can be seen in the table, that those values of S, calculated from Eqns. 2 and 2A, which fulfil this condition (underlined values) are close to 16.

g_z	A	B_{max}	S				
			$B = 0$	$B = 0.04$	$B = 0.08$	$B = 0.12$	$B = 0.16$
3.0	0.866	0.500	15.00	15.36	15.66	15.87	15.99
3.1	0.880	0.474	15.19	15.52	15.77	15.94	16.00
3.2	0.894	0.447	15.36	15.65	15.86	15.98	15.99
3.3	0.908	0.418	15.51	15.76	15.93	16.00	15.95
3.4	0.922	0.387	15.64	15.85	15.98	15.99	15.89
3.5	0.935	0.354	15.75	15.92	16.00	15.96	—
3.6	0.949	0.316	15.84	15.97	16.00	15.91	—
3.7	0.962	0.274	15.91	15.99	15.97	15.83	—
3.8	0.975	0.224	15.96	16.00	15.93	15.73	15.38
3.9	0.987	0.158	15.99	15.98	15.86	15.61	—
4.0	1.000	0.000	16.00	—	—	—	—

columns wherein $2 < C/B < 5$ the sum of the squares of the g values equals or nearly equals 16.

It is a fair approximation then to put:

$$S = g_x^2 + g_y^2 + g_z^2 = 16 \tag{3}$$

if $3 < g_z < 4$.

Integration

The proportionality factor of Aasa and Vänngård [1] can be written as follows:

$$T = \frac{\beta}{h\nu} \cdot \frac{g_x^2 + g_y^2}{2 \cdot \left[1 - \frac{g_x^2 + g_y^2}{g_z^2} + \frac{g_x^2 \cdot g_y^2}{g_z^4}\right]^{1/2}} \tag{4}$$

Substituting $g_x^2 + g_y^2 = 16 - g_z^2$ (Eqn. 3):

$$T' = \frac{\beta}{h\nu} \cdot \frac{16 - g_z^2}{2 \cdot \left[1 - \frac{16 - g_z^2}{g_z^2} + \frac{g_x^2 \cdot g_y^2}{g_z^4}\right]^{1/2}} \tag{5}$$

Put $R = \frac{g_x^2 \cdot g_y^2}{g_z^4}$.

Then, if g_x or g_y equals zero, $R_{\min} = 0$, so:

$$T^{\max} = \frac{\beta}{h\nu} \cdot \frac{16 - g_z^2}{2 \cdot \left[1 - \frac{16 - g_z^2}{g_z^2}\right]^{1/2}} \tag{6}$$

If $g_x = g_y$, then $R_{\max} = \frac{(16 - g_z^2)^2}{4 \cdot g_z^4}$, so:

$$T^{\min} = \frac{\beta}{h\nu} \cdot \frac{16 - g_z^2}{2 \cdot \left[1 - \frac{16 - g_z^2}{g_z^2} + \frac{(16 - g_z^2)^2}{4g_z^4}\right]^{1/2}} \tag{7}$$

Table II shows the values of $T^{\min}$ and $T^{\max}$. Within the approximation that $S = 16$, $T^{\min}$ and $T^{\max}$ represent the extreme values of T at a fixed g_z, independent of g_y and g_x. Since, then, the real value of T is somewhere between $T^{\min}$ and $T^{\max}$, the average is a better approximation than $T^{\min}$ or $T^{\max}$ alone.

$$T^{AV} = \tfrac{1}{2}(T^{\min} + T^{\max}) \tag{8}$$

For practical use T^{AV} can be rearranged to:

$$T^{AV} = \frac{1}{\nu} \cdot \frac{\beta q}{4h}\left[\frac{1}{(1-r)^{1/2}} + \frac{2}{2-r}\right] \tag{9}$$

where $q = 16.018 - g_z^2$ and $r = q/g_z^2$. Note that $g_e = 2.0023$ has been introduced. The intensity of a low-spin heme signal with $g_z > 3$ is then calculated according to Aasa and Vänngård [1], but T^{MI} is replaced by T^{AV}.

TABLE II

CALCULATION OF T^{AV}, T^{min} AND T^{max} AT DIFFERENT VALUES OF g_z

T^{max}, T^{min} and T^{AV} were calculated with Eqns. 6, 7 and 8, in which the factor $1/\nu$ has been omitted and 16 is replaced by 16.018. Deviation is defined as $\pm (1-T^{min}/T^{AV}) \times 100\%$. Note that the deviation decreases with increasing g_z values.

g_z	T^{max}	T^{min}	T^{AV}	Deviation (%)
3.0	10.465	8.050	9.257	± 13.0
3.1	7.769	6.727	7.248	± 7.2
3.2	6.125	5.632	5.879	± 4.2
3.3	4.933	4.694	4.813	± 2.5
3.4	3.980	3.865	3.922	± 1.5
3.5	3.169	3.116	3.142	± 0.8
3.6	2.448	2.426	2.437	± 0.5
3.7	1.788	1.780	1.784	± 0.2
3.8	1.170	1.168	1.169	<0.1
3.9	0.581	0.581	0.581	<0.01
4.0	0.013	0.013	0.013	0

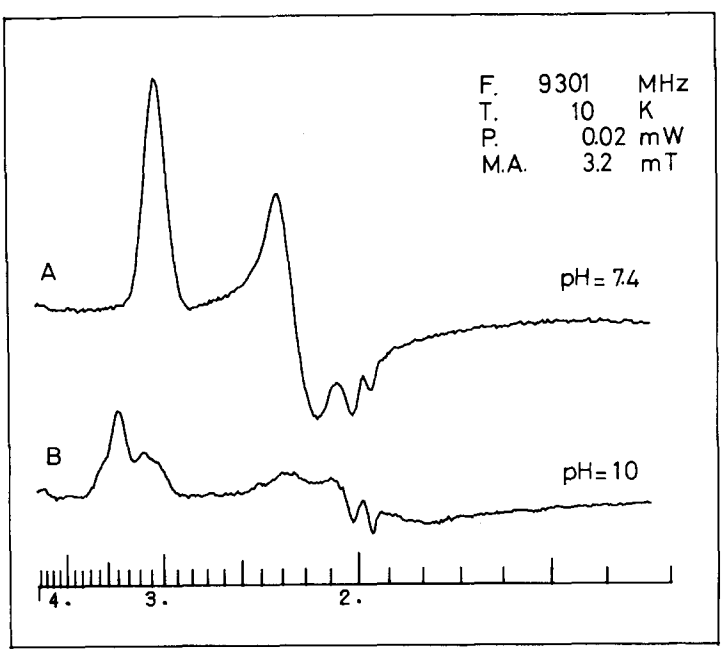

Fig. 1. Comparison of the EPR spectra of cytochrome *c* at pH 7.4 and pH 10. (A) Oxidized cytochrome *c* in 50 mM Tris-HCl buffer (pH 7.4). (B) Oxidized cytochrome *c* in 50 mM Tris-HCl buffer (pH 7.4) titrated with 1 N NaOH until pH = 10. Both samples were diluted to the same extent and the spectra were recorded with the same gain. Only a small fraction of the signal at g_z = 3.06 appears in trace B, and three new signals appear with the following g_z values: 3.16, 3.40 and 3.48. The origin of the sharp signals around g = 2 is not clear, but they are also present in the spectra of Ref. 6. EPR conditions: Frequency (F), 9301 MHz; temperature (T), 10 K; microwave power (P), 20 μW; modulation amplitude (MA), 3.2 mT; scan rate (SR), 100 mT/min. The field modulation frequency for these spectra and those in Fig. 2 is 100 kHz. The scale at the bottom of the figures is of g values.

TABLE III

COMPARISON OF OPTICALLY AND EPR-DETERMINED CONCENTRATIONS OF SOME LOW-SPIN HEME COMPOUNDS WITH HIGHLY ANISOTROPIC EPR SPECTRA

T was computed from Eqn. 4, T^{AV} with Eqn. 9. The intensity of the signal of copper perchlorate served as a standard.

Sample	g_z	g_y	g_x	T	T^{AV}	Concentration (μM)	
						EPR	Optically
Cytochrome *c* at pH 7.4	3.06	2.25	1.25	7.495	—	1286	1330
Cytochrome *c* at pH 10							
Component 1	3.06	2.25	1.25	7.495	—	149	
Component 2	3.159	—	—	—	6.394	292	
Component 3	3.397	—	—	—	3.949	432	
Component 4	3.48	—	—	—	3.293	414	
Total (1 + 2 + 3 + 4)						1287	1330
Cytochrome *c* + cyanide	3.343	—	—	—	4.415	1740	1830
Metmyoglobin + cyanide	3.383	—	—	—	4.066	1950	1980

Validity of Eqn. 9

Fig. 1 shows the EPR spectra of cytochrome *c* at pH 7.4 and pH 10. In a recent article [6] it was shown that the EPR spectrum of cytochrome *c* is pH dependent. Three new signals appear in the spectrum of pH 10 and only the

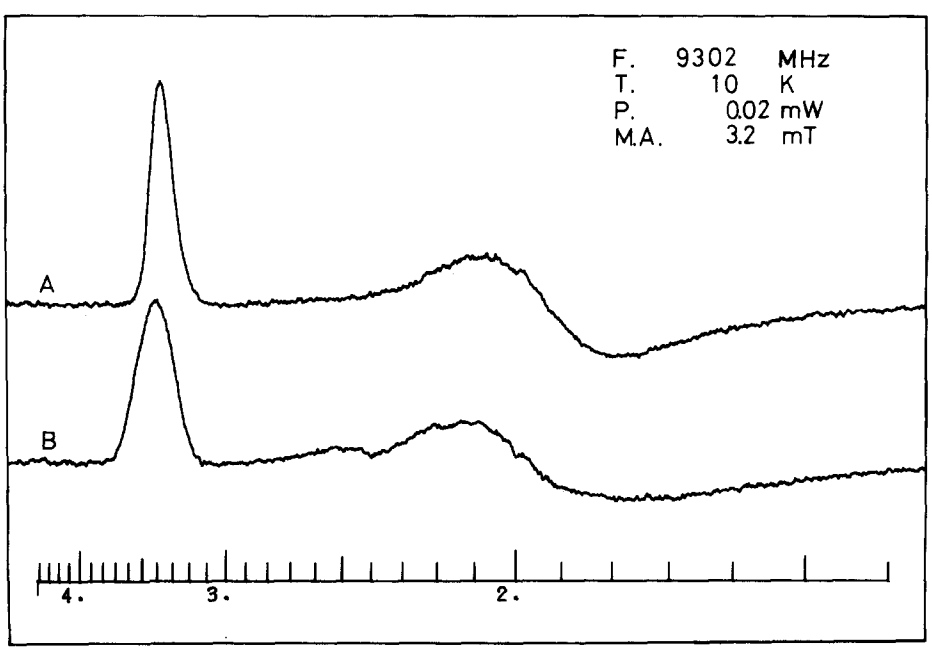

Fig. 2. EPR spectra of the cyanide complexes of cytochrome *c* and metmyoglobin. (A) Oxidized cytochrome *c* in 50 mM Tris-HCl buffer and 140 mM KCN (pH 7.4). The g_z value is 3.343. (B) Metmyoglobin in 50 mM Tris-HCl buffer and 140 mM KCN (pH 7.4). The g_z value is 3.383. For both spectra the g_y value is around 1.9 and g_x values cannot be detected. EPR conditions: F, 9302 MHz; T, 10 K; P, 20 μW; MA, 3.2 mT and SR, 100 mT/min. For symbols see Fig. 1.

g_z peaks are clearly visible. Note that the area under the g_z peaks in trace B is much smaller than in trace A, although both represent the same number of spins. The proportionality factor T^{AV} completely compensates for the difference in area's, since: number of spins = concentration $\approx$ (area)/T^{AV}.

Assuming a Gaussian line shape, the g_z peaks in trace B were resolved into the individual components with a Curve Resolver. The area's, g values and values of T^{AV} of the individual peaks were then used to compute the concentration of each component. The results are listed in Table III. There is a good correlation between the concentration determined by optical and EPR spectrometry.

Fig. 2 shows the EPR spectra of the cyanide complexes of cytochrome *c* and metmyoglobin. According to the literature [6,12] both compounds have the same set of g values: $g_z = 3.45$, $g_y = 1.89$ and $g_x = 0.93$. However, we measured slightly different g values (see the legend of Fig. 2) and in neither case could we detect the g_x line. The concentration was calculated with Eqn. 9 and compared with the optically determined concentration of both compounds in the absence of cyanide and corrected for dilution with cyanide. The results are shown in Table III.

Discussion

The only assumption made in the derivation of Eqn. 9 was that S equals 16 if $3 < g_z < 4$. From a purely theoretical viewpoint this assumption cannot be proven and so Eqn. 9 is a semi-empirical equation. However, Table I shows that in all cases the maximum of 16 can be reached and that it appears from experimental data that the ratio of C and B is such that the maximum of 16 is attained for the low-spin heme compounds with $g_z > 3.0$. If this ratio remains fixed for other low-spin heme compounds with highly anisotropic EPR spectra, the sum of the squares of the g values is also 16 for these compounds. If one assumes that $k < 1$ then $S < 16$. The values of T^{AV} then become smaller and the computed concentrations greater. However, the close correspondence between the optically and EPR-determined concentrations justifies the assumption that S equals 16.

Note that the difference between T^{min} and T^{max} decreases with increasing g_z values and that if g_z is greater than 3.3 this difference is well within experimental error. For cytochrome *c*, with $g_z = 3.06$, the difference between T^{min} and T^{max} is 18%. The difference between T^{AV} and T is, however, only 5.9% and this makes T^{AV} a better approximation then either T^{min} and T^{max}. It is of course preferable to use the expression of Aasa and Vänngård when the three g values are known. If only one or two g values can be detected T^{AV} gives a very good approximation of T.

This approximation becomes even better at greater g_z values. In practice it appears that in this case the g_y and/or g_x resonances cannot be detected and only Eqn. 9 can be used to calculate the proportionality factor and so the concentration.

Eqn. 9 was especially developed to determine the stoichiometry of the several heme groups in QH_2: cytochrome *c* oxidoreductase, that have highly anisotropic EPR spectra with g_z values greater than 3.3 [13,14]. The results of these studies are presented in an accompanying paper [15].

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