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The magnetic susceptibility of native soybean lipoxygenase-1. Implications for the symmetry of the iron environment and the possible coordination of dioxygen to Fe (II)

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(1) The magnetic susceptibility of native soybean lipoxygenase-1, a non-heme iron dioxygenase, has been measured over the temperature range 10–170 K. (2) With the ground-state multiplet of its high-spin Fe(II) described by means of a spin Hamiltonian, the temperature dependence of the susceptibility is found to be consistent with the zero-field splitting parameters, D and E, within the limits $D = 8.5$ cm^{-1}, $|E/D| = 0.14$ and $D = 11.9$ cm^{-1}, $|E/D| = 0$. The best agreement with the experimental data was obtained for $D = 11.1$ cm^{-1} and $|E/D| = 0.04$. Less satisfactory agreement could be obtained with negative D values in the range -6.9 to -5.5 cm^{-1} and $|E/D| < 0.01$. These values are compatible with an iron environment of largely axial symmetry, possibly with a small rhombic distortion. (3) No evidence could be found from the magnetic studies (in the temperature range 27–170 K) that dioxygen should be coordinated to the iron of the native enzyme form.

Introduction

Lipoxygenase (linoleate : oxygen oxidoreductase, EC 1.13.11.12), a non-heme iron dioxygenase, catalyzes the dioxygenation of polyunsaturated fatty acids containing a 1,4-*Z*,*Z*-pentadiene system (1). The main product of an incubation of linoleic acid with soybean lipoxygenase-1 (M_r 98 500) at pH 9.0 is 13-L$_s$-hydroperoxy-(9*Z*,11*E*)octadecadienoic acid (13-L-HPOD) (2).

Soybean lipoxygenase-1 contains one gatom of iron per mol enzyme. The iron is supposed to cycle between the Fe(II) and Fe(III) states during the catalytic reaction [3].

In native lipoxygenase-1, the iron was found to be in the high-spin Fe(II) state ($S = 2$) from NMR spectroscopic and magnetic susceptibility studies [4]. It has been suggested that dioxygen is one of the iron ligands in this form [3]. Upon addition of one molar equivalent of 13-L-HPOD to the native colorless enzyme, a yellow form is obtained. Approximately 75% of its iron is EPR-visible and was found to be in the high-spin Fe(III) state ($S = \frac{5}{2}$) with a ligand field of largely axial symmetry [5]. Since the native enzyme is virtually EPR-silent, no information could be deduced on the symmetry of the iron environment in this form. Furthermore, Mössbauer studies could not be carried out because of serious difficulties with the incorporation of ^{57}Fe [6].

Our magnetic susceptibility studies on native lipoxygenase-1 have now been extended down to 10 K in order to allow a determination of the axial

Abbreviation: 13-L-HPOD, 13-L$_s$-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid.

and rhombic zero-field splitting parameters (D and E, respectively), which provide information on the symmetry of the iron environment. Furthermore, the effect of dioxygen on the magnetic susceptibility of the native enzyme has been investigated.

Materials and Methods

Soybean lipoxygenase-1 was isolated according to Slappendel [7]. The specific activity was 235 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$, corresponding to 3.92 μkat $\cdot mg^{-1}$. The amounts of iron and contaminating manganese were 0.97 and 0.07 mol per mol enzyme, respectively [5]. In native lipoxygenase, the amount of EPR-detectable high-spin Fe(III) was less than 0.01 mol per mol enzyme, which is conceived as a contaminant [5]. Prior to the magnetic susceptibility measurements, the enzyme was dialyzed against 0.1 M sodium borate buffer (pH 9.0) and concentrated in a Collodion-Bag SM 13200 (Sartorius Membranfilter, Göttingen, F.R.G.). The enzyme concentration was determined from the absorbance at 280 nm using $A_{280}^{0.1\%} = 1.6$ with an estimated accuracy of $\pm 5\%$.

Magnetic susceptibility measurements were made with a highly sensitive magnetic balance of the Faraday type [8,24]. This instrument can resolve a change in the susceptibility which is larger than $2 \cdot 10^{-10}$ c.g.s. e.m.u./cm^3 for a 0.1 cm^3 sample of a protein solution. The accessible temperature range is presently 10–200 K [24].

Results and Discussion

Determination of the Bohr magneton number

The results of the magnetic susceptibility measurements are shown in Fig. 1, where the temperature-dependent contribution of the molar susceptibility is displayed as a function of inverse temperature for deoxygenated native soybean lipoxygenase-1. The contribution from the protein was obtained as the difference between two series of measurements, i.e., one from the protein solution and the other from a blank. The blank used was a buffer solution taken from a final stage of the sample preparation. The curve fitted to the experimental points was obtained by a computer least-squares procedure as described below. The straight

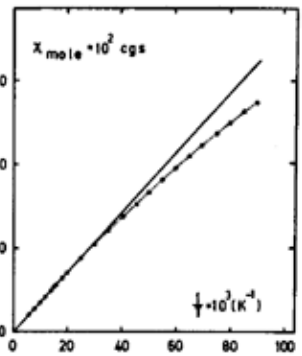

Fig. 1. The temperature-dependent contribution of the molar magnetic susceptibility (as a function of inverse temperature) for native soybean lipoxygenase-1, 97 μl, 1.28 mM in 0.1 M sodium borate buffer (pH 9.0). The curve was fitted to the experimental data by means of a non-linear least-squares computer procedure (Biomedical computer programs: BMDPAR), resulting in $D = 11.1$ cm^{-1} and $|E/D| = 0.04$. Spin-only contributions were assumed from the contaminating iron and manganese. An average value of 16.3 kOe was used for the applied magnetic field. The straight line represents the best fit to the experimental data obtained above 50 K.

line represents the best fit to the experimental points above 50 K, where the susceptibility shows Curie behaviour ($\chi \propto 1/T$). After correction for the paramagnetic contributions of the contaminating iron (high-spin Fe(III)) and manganese (probably high-spin Mn(II)) as described previously [4], a Bohr magneton number of 5.1 was calculated from the slope of the line. This is in good agreement with the value 5.2 obtained for a non-deoxygenated sample [4] and consistent with the presence of one high-spin Fe(II) per enzyme molecule. Recently the high-spin state of Fe(II) in native lipoxygenase has also been confirmed by ^{1}H-NMR shift experiments [9].

Determination of the axial and rhombic zero-field splitting parameters

Fe(II) has six valence electrons distributed over the five 3*d* orbitals. For the free ion, Hund's rules

predict a ^5D electronic ground state, which is 25-fold degenerate.

The interaction between the electrons and the crystal field (the electric field produced at the ion by its neighbours) separates these levels into five spin quintet states. Provided that the ground state quintet is sufficiently separated from the excited quintet states, it can be described by means of a spin Hamiltonian [10,11]. A fictitious spin operator is then introduced operating on spin-dependent wave functions only. The spin Hamiltonian requires little information about the system treated and allows the electronic energy levels to be expressed by means of a small number of parameters. For native lipoxygenase this approach seems to be justified, since there is no evidence from magnetic susceptibility studies up to 200 K that excited states should be thermally accessible [4].

The actual spin Hamiltonian is then given by:

$$\mathcal{H} = D\left[S_z^2 - \tfrac{1}{3}S(S+1)\right] + E\left[S_x^2 - S_y^2\right] + g\beta\vec{H}\cdot\vec{S}$$

where D and E are the axial and rhombic zero-field splitting parameters, respectively, g is the electronic g value of the iron, which is approximated to be isotropic and equal to the free electron g value $g_e = 2$. S is the fictitious spin quantum number, which for high-spin Fe(II) equals 2. β is the Bohr magneton and H is the applied magnetic field. The electronic energy levels, E_i, of the ground state were calculated by means of second-order perturbation calculation, supposing that $D \gg E$ and $D \gg g\beta H$. The basis set $|M_s>$ ($M_s = 2, 1, 0, -1, -2$) was used.

The molar susceptibilities with the external field applied along the x, y and z directions, respectively, were obtained from the energy levels by (cf. Ref. 12):

$$\chi_j^{mole} = \frac{N}{H}\frac{\sum_{i=1}^{5} -\frac{\partial E_{ij}}{\partial H} e^{-E_{ij}/kT}}{\sum_{i=1}^{5} e^{-E_{ij}/kT}}$$

where $j = x, y, z$. Since the molecules of the frozen solution are randomly oriented, an averaged value over all directions of the applied field is required. This average was approximated to be:

$$\chi_{av}^{mole} = \tfrac{1}{3}\sum_{j=x,y,z}\chi_j^{mole}$$

The force contribution, ΔF, in the Faraday balance from the iron is obtained as:

$$\Delta F = m \cdot \chi_{av}^{mole} \cdot H\frac{\partial H}{\partial z}$$

where m is the number of moles of high-spin Fe(II) in the sample and $H\frac{\partial H}{\partial z}$ the product of the applied horizontal magnetic field and its vertical gradient. The theoretical expression, fitted to the experimental points, contained in addition two other terms. A temperature-independent term was added to account for contributions of diamagnetic origin (cf. Ref. 13) and a Curie-dependent contribution was supposed to originate from the contaminating iron and manganese (cf. Ref. 4). Consequently, the metal contaminations were supposed not to contribute to the deviation from Curie behaviour. This is reasonable, since the amount of high-spin Fe(III) is very small (less than 0.01 mol per mol enzyme). Furthermore, the EPR-detectable high-spin Mn(II) of the native enzyme [5] apparently is in a slightly distorted cubic environment as indicated by its EPR-signal around g 2 (cf. Ref. 14). The zero-field splitting parameters should then be very small. Observed D values in similar cases are generally of the order of 0.01 cm^{-1} [15].

An optimal fit, i.e., a minimum value of the error mean square (cf. Ref. 8), was obtained with $D = 11.1$ cm^{-1} and $|E/D| = 0.04$. Fits with an error mean square less than twice the value found in this case were accepted. In the limits, the parameter values were found to be $D = 8.5$ cm^{-1}, $|E/D| = 0.14$ and $D = 11.9$ cm^{-1}, $|E/D| = 0.0$, respectively. Less satisfactory fits could be obtained with negative D values. With the acceptance criterion given above, fits with D in the range -6.9 to -5.5 cm^{-1} and $|E/D| < 0.01$ would, however, be acceptable. The values found for the zero-field splitting parameters are consistent with an iron environment of largely axial symmetry, possibly with a small rhombic distortion [16].

A positive value of D has also been found for high-spin Fe(III) in the yellow form of soybean lipoxygenase-1 ($D = 1.9\ cm^{-1}$) (mean value)) [6].

For another non-heme iron containing dioxygenase, protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*, zero-field splitting parameters of different enzyme forms have been reported. Thus a positive D value ($D = 1.5\ cm^{-1}$) was found for high-spin Fe(III) in the native enzyme [17,18], whereas negative D values were found for high-spin Fe(II) in the reduced enzyme ($D = -6\ cm^{-1}$) [19] and in a ternary enzyme-substrate complex ($D \approx -2\ cm^{-1}$) [18].

Possible coordination of dioxygen to Fe(II)

For native lipoxygenase it has been suggested that dioxygen could be coordinated to the iron [3, 20–22]. To explore this possibility further, the effect of deoxygenation was investigated. Thus, the susceptibility of the present sample was measured in the temperature range 27–170 K before any attempts to remove dioxygen. Subsequently, the sample was reinvestigated in the same temperature range after deoxygenation at 4°C. The deoxygenation was performed directly in the sample container by flowing humidified pure argon gas over the sample surface for about 40 min while stirring (cf. Ref. 8). Deoxygenated, twice-distilled water was used in the gas wash-bottles. To ensure the removal of dissolved dioxygen, a second deoxygenation was carried out with the wash-bottles containing $Na_2S_2O_4$ (20 g/l) in 0.1 M NaOH, whereupon the results of Fig. 1 were obtained.

No significant differences were observed between the aerobic and anaerobic samples, neither as concerns the Bohr magneton number nor the deviation from Curie behaviour down to 27 K. At the lowest temperature, the deviation from Curie behaviour is of the order of 20-times the resolution of the instrument. It is unlikely that this result is due to poor deoxygenation, since the deoxygenation procedures employed have been thoroughly tested. The possibility that dioxygen is so firmly coordinated to the iron of the native enzyme that it is not removed by the deoxygenation procedures is also unlikely. In analogy with the oxy forms of hemoglobin and myoglobin, a diamagnetic ground state could be anticipated for stable dioxygen liganded Fe(II) [23]. In contrast, our experimental results show a strongly paramagnetic iron.

Then the possibilities remain that dioxygen is either not coordinated to the iron of the native enzyme or attached so weakly that the electronic state of the iron is not significantly affected.

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References

1 Vliegenthart, J.F.G. and Veldink, G.A. (1982) in Free Radicals in Biology (Pryor, W., ed.), Vol. 5, pp. 29–64, Academic Press, London
2 Hamberg, M. and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329–5335
3 De Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and Van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71–79
4 Slappendel, S., Malmström, B.G., Petersson, L., Ehrenberg, A., Veldink, G.A. and Vliegenthart, J.F.G. (1982) Biochem. Biophys. Res. Commun. 108, 673–677
5 Slappendel, S., Veldink, G.A., Vliegenthart, J.F.G., Aasa, R. and Malmström, B.G. (1981) Biochim. Biophys. Acta 667, 77–86
6 Slappendel, S., Veldink, G.A., Vliegenthart, J.F.G., Aasa, R. and Malmström, B.G. (1980) Biochim. Biophys. Acta 642, 30–39
7 Slappendel, S. (1982) Thesis, University of Utrecht, Utrecht, The Netherlands
8 Petersson, L. (1981) Thesis, University of Stockholm, Stockholm, Sweden
9 Cheesbrough, T.M. and Axelrod, B. (1983) Biochemistry 22, 3387–3840
10 Agragam, A. and Bleaney, B. (1970) Electron Paramagnetic Resonance of Transition Ions, Clarendon Press, Oxford
11 Agragam, A. and Pryce, M.H.L. (1951) Proc. Roy. Soc. A205, 135–153
12 Van Vleck, J.H. (1932) The Theory of Electric and Magnetic Susceptibilities, Oxford University Press, London
13 Petersson, L., Cammack, R. and Rao, K.K. (1980) Biochim. Biophys. Acta 622, 18–24
14 De Wijn, H.W. and van Balderen, R.F. (1967) J. Chem. Phys. 46, 1381–1387

15 Dowsing, R.D., Gibson, J.F., Goodgame, D.M.L., Goodgame, M. and Hayward, P.J. (1968) Nature 219, 1037–1038
16 Orton, J.W. (1968) Electron Paramagnetic Resonance. An Introduction to Transition Group Ions in Crystals, Iliffe Books, London
17 Blumberg, W.E. and Peisach, J. (1973) Ann. N.Y. Acad. Sci. 222, 539–560
18 Que, L., Jr., Lipscomb, J.D., Zimmermann, R., Münch, E., Orme-Johnson, N.R. and Orme-Johnson, W.H. (1976) Biochim. Biophys. Acta 452, 320–334
19 Zimmermann, R., Huynh, B.H., Münck, E. and Lipscomb, J.D. (1978) J. Chem. Phys. 69, 5463–5467
20 Finazzi-Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 462–470
21 Finazzi-Agrò, A., Avigliano, L., Egmond, M.R., Veldink, G.A. and Vliegenthart, J.F.G. (1975) FEBS Lett. 52, 73–76
22 Veldink, G.A., Garssen, G.J., Slappendel, S., Vliegenthart, J.F.G. and Boldingh, J. (1977) Biochem. Biophys. Res. Commun. 78, 424–428
23 Thomson, A.J. (1977) Nature 265, 15–16
24 Petersson, L. and Ehrenberg, A. (1985) Rev. Sci. Instr., in the press