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THE INTERACTION OF NITRIC OXIDE WITH SOYBEAN LIPOXYGENASE-1

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Summary

The interaction of nitric oxide with the non-heme iron dioxygenase lipoxygenase is reported. This apparently resulted in a novel type of complex where an electron is donated to the NO molecule. In addition a new position for an EPR transition from iron was discovered which, it is suggested results from high spin ferric iron in a field of axial symmetry characterised by a very low value for D.

Introduction

Soybean lipoxygenase-1 (linoleate:oxygen oxidoreductase EC 1.13.11.12) contains 1 atom of non-heme iron per mol wt. 98 500. It catalyses the following type of reaction:

$$R{-}CH \overset{cis}{=} CH{-}CH_2{-}CH \overset{cis}{=} CH{-}R' + O_2 \rightarrow$$

$$R{-}\underset{\underset{OOH}{|}}{CH}{-}CH \overset{trans}{=\!=} CH{-}CH \overset{cis}{=} CH{-}R'$$

the product from linoleic acid being 13-L-hydroperoxy-*cis,trans*-9,11-octadecadienoic acid (where R is $CH_3(CH_2)_4$, R′ is $(CH_2)_7COOH$); Compound I).

Nitric oxide is a paramagnetic molecule whose bonding structure is very similar to that of molecular oxygen. It has been used on a number of occasions as an alternative to molecular oxygen, which is EPR silent, as a probe for investigating aspects of the binding of oxygen to metals in biological systems. The majority of these systems were heme-containing proteins e.g. myoglobin, haemoglobin, cytochrome oxidase and catalase [1,2,3]. One characteristic feature of these experiments is that an EPR signal was only observed when the iron atom was initially present in the ferrous (diamagnetic) form. Addition to a

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ferric form probably results in a diamagnetic $Fe^{3+} \cdot N = O$ complex, perhaps with the transfer of the unpaired electron from the nitrogen to the iron [2]. Another feature of those complexes which show an EPR signal i.e. $Fe^{2+} \cdot NO$ is that the signal found is always from the nitroxide moiety. There seems to have been no reported biochemical instance of transfer of an electron to give Fe^{3+}-NO which would also be paramagnetic but show a signal from the iron atom. We report just a case with the appearance of a new position for an iron EPR signal.

Materials and Methods

Soybean lipoxygenase-1 was isolated as previously reported [4] and had a specific activity 240 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ when assayed at 25°C with 1.8 mM linoleic acid as substrate dissolved in 0.1 M sodium borate buffer (pH 9.5).

Linoleic acid was from Lipid Supplies, Dept. of Chemistry, University of Glasgow, U.K., purity 99%.

13—Hydroxy-*cis,trans*-9,11-octadecadienoic acid (Compound I) was prepared enzymically from this linoleic acid and purified by high performance liquid chromatography [5].

Enzyme samples for EPR were frozen in quartz tubes to liquid N_2 temperature. They were then repeatedly evacuated with a rotary oil pump and flushed with high purity N_2 or A to ensure complete removal of O_2. They were then sealed with a silicon rubber bung pierced by a syringe needle. This was then attached to a three-way system, which allowed evacuation or flushing with NO gas (Baker lecture bottle). EPR spectra were recorded on a Varian E4 spectrometer equipped with an Oxford Instruments liquid helium cryostat and control system. Anaerobic enzyme assays were performed on a Cary 118C spectrophotometer by monitoring absorption at 234 nm.

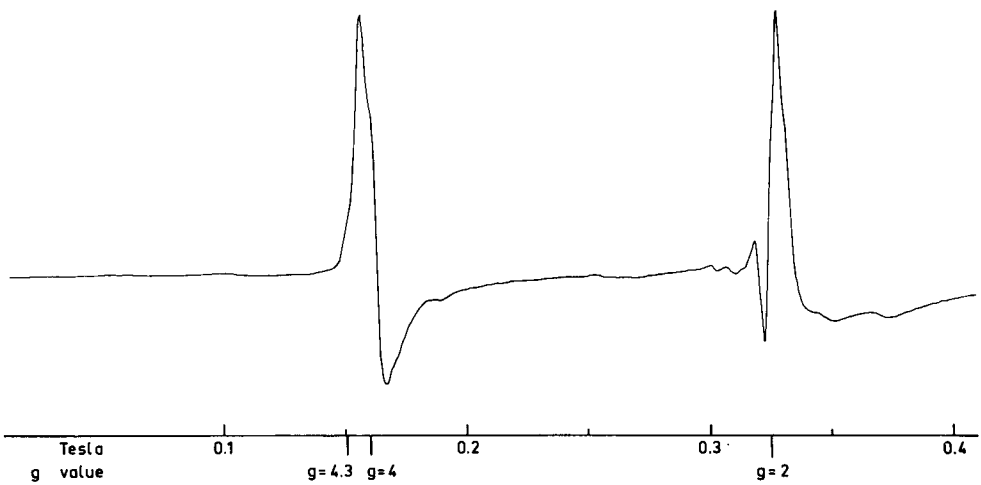
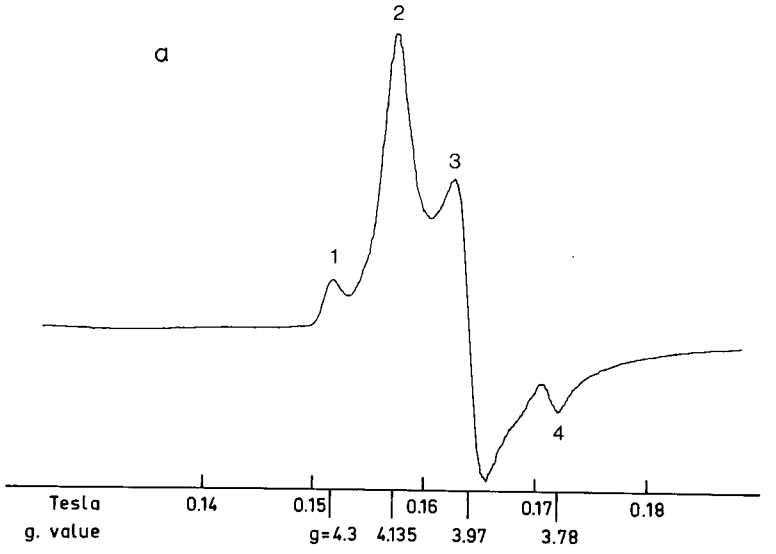


Fig. 1. Spectrum after the addition of NO gas to anaerobic native lipoxygenase 85 mg $\cdot$ ml^{-1} in 0.1 M sodium phosphate (pH 7.0). 9 $\cdot$ 105 GHz microwave frequency; 0.5 mW power; receiver gain 10^3; temperature 15.3 K.



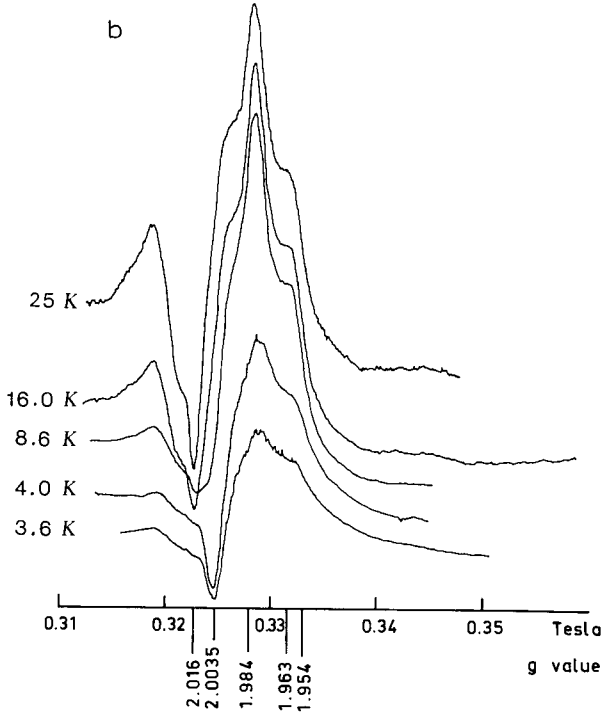


Fig. 2. (a) '$g \approx 4$'. transition from Fig. 1 with 0.1 mW Microwave power. Receiver gain $4 \cdot 10^2$; temperature 3.7 K. (b) '$g \approx 2$' transition from Fig. 1. The receiver gain was varied with temperature such that if Curie law was obeyed all signals should remain the same size.

Results

It was first determined whether NO could replace O_2 in the normal reaction. Oxygen-free 0.1 M sodium borate buffer (pH 9.5) was saturated with NO gas. This was then transferred to sealed O_2-free cuvettes. Linoleic acid (final concentration up to 100 μM) and lipoxygenase (final concentration up to 1 μM) where then added by use of micro-syringes. No changes in A_{234} could be detected for up to 30 min at 25°C.

When NO gas was introduced to concentrated anaerobic samples of the native enzyme to be used for EPR at either pH 7.0 in 0.1 M phosphate of pH 9.5 in 0.1 M borate there was an immediate appearance of a pale yellow colour. This appears to be essentially identical to that of the ferric enzyme form found on treating the native enzyme with 1 equivalent product (Compound I) [7].

A typical EPR spectrum of this lipoxygenase · NO complex at 15 K is shown in Fig. 1. The feature near $g = 4$ showed no departure from Curie dependence on cooling to 3.7 K although a more detailed structure could be discerned (Fig. 2a). The feature around $g = 2$ however decreased markedly on cooling below about 8 K (Fig. 2b).

Addition of linoleic acid in large excess (10 mM, final concentration) caused no discernable change to the signal near $g = 4$ however some change was seen in the signal around $g = 2$ (Fig. 3).

However, the addition of Compound I to the native enzyme · NO complex results in a substantial decrease in the signal near $g = 4$. In addition a total change is seen in the structure near $g = 2$ (Fig. 4, the difference in spectrometer gain should be noted). There was also some loss of colour. The appearance of the radical type of signal in this region was no reproducible but there was always a total loss of the other absorption. If Compound I was added before

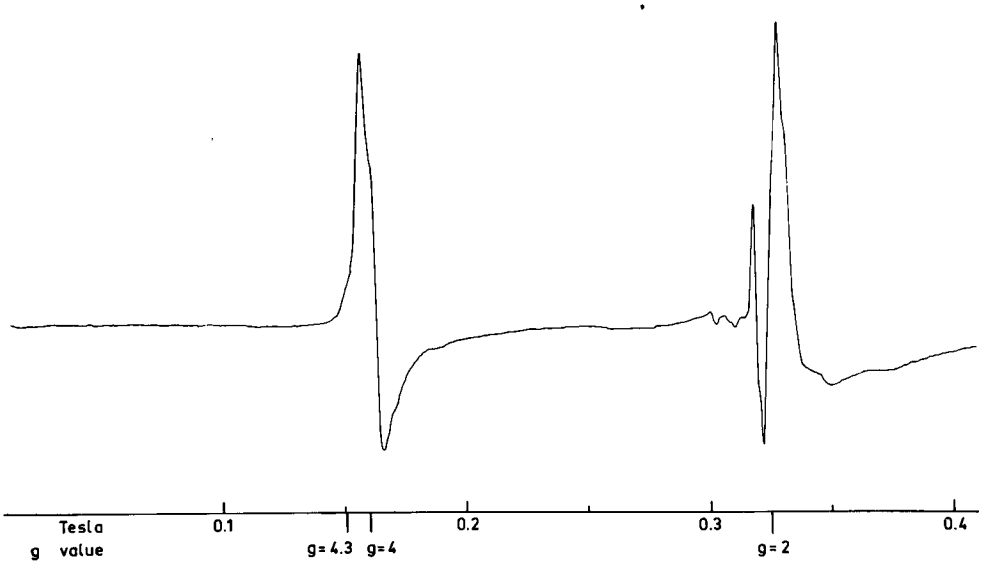


Fig. 3. As Fig. 1 but with the addition of ammonium linoleate to give a final concentration of 0.8 M enzyme and 8 mM linoleate.

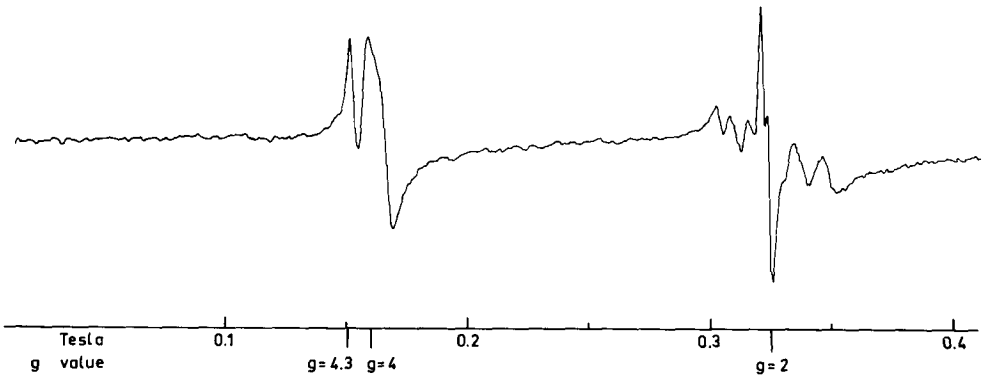


Fig. 4. As Fig. 1 but with the addition of Compound I in a 2 : 1 ratio to enzyme. Conditions as Fig. 1 except receiver gain = $5 \cdot 10^3$.

treatment with NO, the signal seen near $g = 6$ [7] disappeared completely and only a relatively small signal near $g = 4$ was seen. (There was some loss of colour). Unlike other ferrous · NO complexes, no EPR signals could be seen at room temperature when the experiments were conducted in a solution cell.

The formation of the native enzyme · NO complex appeared to be essentially reversible by evacuation. The yellow colour and typical EPR signal were lost and over 90% of the enzyme activity in the normal aerobic reaction was retained.

Discussion

The first unique feature of this system is that an electron has been transferred from the iron atom to an NO molecule, it being inconceivable that the signal near $g = 4$ be ascribed to a nitroxide radical but must originate from high spin ferric iron. The signal near $g = 4$ is also, we believe, quite unusual. Normally signals near this region, the so-called $g = 4.3$ signals, are ascribed to iron in a ligand field of rhombic symmetry. The signal we observe however has the wrong lineshape, fine structure and precise position to be ascribed to such a species. In fact its form and structure (lines 2 and 3, Fig. 2a) is almost identical with that which would be expected from an almost axial ligand field ($E/D \sim 0$) e.g. similar to that seen in the Fe(III) yellow lipoxygenase, which occurs around $g = 6$. However, examination of the data presented by Aasa [8] allows one to see that at very small values of D (the axial tensor coefficient), approximately 0.15 K, the normal $g = 6$ signal of an axial ligand field around a ferric iron atom is expected to be seen near $g = 4$ when using an X band ($\approx$9.1 GHz) instrument.

The general principle of this can be seen by examining the spin Hamiltonian which is usually adequate to describe high spin ferric iron.

$$\mathcal{H} = D[(S_z^2 - \tfrac{35}{12} + 1)(S_x^2 - S_y^2)] + g_0 B\vec{S}\vec{H}$$

The first term describes the splitting of the spins into non degenerate doublets (Kramers doublets) and the influence of directional symmetry elements on EPR line shape and position as a result of interaction with ligand electrons (the

ligand field). The second term describes the interaction of the electron magnetic moment with the applied magnetic field. In most biological systems the first term is greater than the second when using instruments at <35 GHz. However, when D, the axial field coefficient becomes small the line positions tend towards the free spin value given by the second term and become much more frequency dependent but with axial symmetry their shape remains generally unaltered. Line 1 in Fig. 2a is probably from a small amount of contaminating iron. With such a small D value it could however originate from an absorption between the upper two Kramers doublets. Similarly, line 4 may well come from a transition between the lowest two Kramers doublets.

The signal just below $g = 2$ is much more difficult to explain. Its size appears somewhat variable in comparison with that of the $g = 4$ signal (approx. ±20%). Unlike ferro heme · NO complexes, no trace of any signal can be seen from the NO moiety of the lipoxygenase · NO complex at room temperature in a solution cell. In the light of this it was suggested to us that this signal could be due to non specific adsorption of NO to the enzyme (Wever, R., personal communication). However, the position of the component lines are all at $g < 2$ which implies a significant orbital contribution more characteristic of that gained in association with a paramagnetic metal. The specific effect of compound I in removing this signal, as opposed to the effect of linoleic acid, suggests that this signal originates from an NO molecule liganded very close to the normal site for oxygen. The negative Curie dependence of these signals at low temperature is in agreement with this [9,10]. This makes it unlikely that non-specific adorption of NO is involved.

The appearance of radical type signals near $g = 2$ in some of our spectra and those described by others [6,11] together with the fact that linoleoyl radicals can be trapped during lipoxygenase catalysed reactions [11] needs some comment. It is not certain whether the signals we see originate from fatty acid radicals or if they are sited on the enzyme itself. This still requires some further investigation.

It seems quite reasonable to conclude from this study that the anaerobic native enzyme is a ferrous species. Others [6] have suggested that the enzyme is an EPR silent ferric form not involving the binding of oxygen, as $Fe(III)O_2^-$, since this is inevitably diamagnetic [10]. Such a suggested paramagnetic EPR silent ferric form would become a diamagnetic EPR silent form on binding of NO.

Acknowledgements

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