ON THE INTERACTION OF SOME CATECHOL DERIVATIVES WITH THE IRON ATOM OF SOYBEAN LIPOXYGENASE

John R. GALPIN, Lodewijk G. M. TIELENS, Gerrit A. VELDINK, Johannes F. G. VLIEGENTHART and Jan BOLDINGH

Department of Organic Chemistry, Rijksuniversiteit van Utrecht, Croesestraat 79, Utrecht, The Netherlands

Received 26 July 1976

1. Introduction

Soya bean lipoxygenase-1 (linoleate: oxygen oxidoreductase, EC 1.13.11.12) contains 1 atom of non-heme iron per mol. wt. 98 500 [1,2]. The iron in the native enzyme is EPR silent. Aerobic addition of substrates or product or the anaerobic addition of product leads to the appearance of axial or rhombic type signals depending on the precise conditions [1–3].

Most iron liganding or chelating agents have been generally ineffective inhibitors of the enzyme and so far none have been shown to quantitatively remove the iron from the enzyme. Toluene-3,4-dithiol and dithizone have been reported as being effective inhibitors [3].

Some catechol derivatives have been suggested as inhibitors of lipoxygenase action but were considered to act as free radical traps. Recently 4-nitro catechol was suggested as a general inhibitor for non-heme iron dioxygenases [4]. We report here on the ability of this and a number of related compounds to inhibit lipoxygenase and to change the EPR signal of the iron atom.

2. Materials and methods

Lipoxygenase was isolated from soya beans according to Finazzi-Agrò et al. [5] except that the beans were swollen in 4 mM disodium EDTA and that 0.5 mM EDTA with 0.1 mM sodium diethyldithiocarbamate was present in all the purification stages. Specific activity: 280 μMO_2 min^{-1} mg^{-1}.

Metal content, as determined by flameless atomic absorption spectroscopy (mole metal/mole enzyme; mol. wt. 98 500). Fe = 0.912; Cu = 0.0018; Mn = 0.086; Ca = 0.21; Mg = 0.18; Zn = 0.03; Co, V, Mo, Ni were undetectable. Linoleic acid was from Lipid Supplies, St. Andrews, Scotland (> 99% pure). 13-L-Hydroperoxy linoleic acid (13-L-ROOH) was prepared enzymically immediately before use [6]. The free Fe (III) yellow form of the enzyme was prepared by stoichiometric addition of 13-L-ROOH as previously reported but with the use of 0.1 M sodium borate buffer pH 9.5 [2]. At pH 7.0 this form was prepared in situ immediately before use. EPR experiments were conducted as previously reported [1]. Enzymic assays were conducted in a Gilson oxygraph using 1.8 mM linoleic acid in 0.1 M sodium phosphate below pH 8.0 and 0.1 M sodium borate above pH 8.0. 4-Nitro catechol was from Baker, all other catechols used were the purest grade commercially available or had been synthesised in this department.

3. Results

3.1. *Inhibition studies*

Incubation of the native (EPR silent) form of the enzyme (1 mg/ml $\sim 10^{-2}$ mM) with 1 mM of the following compounds (A–H) at pH 6.9 or 9.5 for periods of up to 48 h at 4°C either aerobically or

Correspondence to: J. Galpin.

Abbreviations: 13-L-ROOH, 13-L-Hydroperoxy linoleic acid; 4 NC, 4-Nitro Catechol. Enzyme: Linoleate: Oxygen oxidoreductase EC 1.13.11.12.

anaerobically caused no loss of activity of the enzyme on dilution of > 5000 : 1 into an assay at pH 6.9 or 9.5. A – 4 Nitro catechol; B – 1,2-dihydroxybenzene-3,5-disulphonate (Tiron); C – Caffeic acid; D – Protocatechualdehyde; E – Histazine (2;3-Dihydroxyanthraquinone); F – Catechol; G – 7,8-Dihydroxy-4-methyl coumarin; H – 3,4-Dihydroxybenzonitrile.

Incubations of the Fe (III) enzyme at either pH 6.9 or pH 9.5 with A, D, E, F and H in just over stoichiometric proportions gave complete (> 99%) inhibition of the enzyme. At pH 6.9 and 0°C this was virtually instantaneous but required about 30 min at pH 9.5. The slower rate at pH 9.5 may be because some catechols form reversible complexes with boric acid [7].

This inhibition proved impossible to reverse by dialysis at pH values from 4.5 to 10.0 for periods of up to 2 weeks. We demonstrated that there was no significant removal of the iron from the enzyme. With A and E the enzyme retained the very characteristic colour of these substances. Compounds B, C and G caused relatively little (< 20%) inhibition of the enzyme even after incubation for 24 h at 4°C. When, especially at lower pH values, 4 NC is present in the assay at concentrations above 1.0 μM a progressive inactivation of the enzyme is seen and the reaction ceases well before exhaustion of the oxygen.

3.2. *EPR spectra of enzyme–catechol complexes*

The EPR spectrum of the native enzyme is unchanged for 24 h under aerobic or anaerobic conditions by the addition of the catechols used. On addition of the inhibitory catechols A, D, E and F the characteristic $g = 6$ signal of the Fe III (yellow) enzyme is rapidly transformed to a $g = 4.3$ signal (fig.1b, c and d and e respectively). Essentially the same results were obtained aerobically and anaerobically. The changes took longer to take place at pH 9.5 than pH 6.9. Compounds B, C and G had no significant effect on the EPR spectrum. In the case of 4 NC and, slightly less clearly, protocatechualdehyde the $g = 4.3$ signal is sharply split and two other new signals are seen at $g = 5.45$ and $g = 8.1$. Cooling the 4 NC enzyme complex to 5.5°K (fig.2) caused approximately proportional increases in the signals at lower field than $g = 4.2$ at x-band and they are thus all related to the same paramagnetic species. The splitting of the $g = 4.3$ seen in the 4 NC enzyme complex is absolutely dependent on the presence of 13-L-ROOH.

Alterations in the ratio of 4 NC: 13-L-ROOH cause significant changes in the relative heights of these peaks (shown, H scale magnified, in fig.3). Allowing the system to stand for 2 days at 4°C causes autocatalytic destruction of the 13-L-ROOH [8] and disappearance of the splitting (fig.4a). Anaerobic addition of linoleic acid or its diynoic acid analogue (fig.4) (further details of the effects of the diynoic acid on lipoxygenase

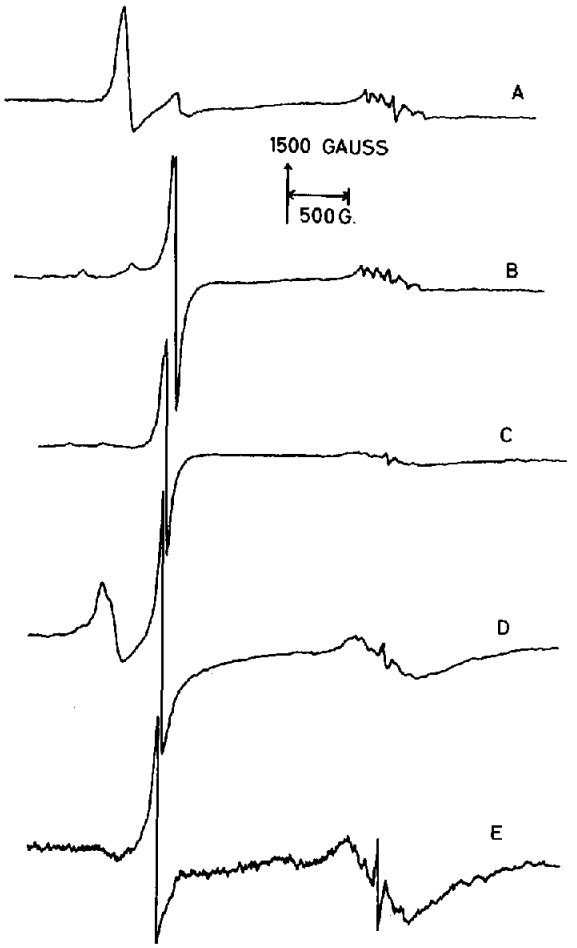

Fig.1(a). EPR spectrum of the native enzyme 50 mg/ml plus stoichiometric addition of 13-L-ROOH in 0.1 mM sodium phosphate, pH 7.0, 15°K. Microwave frequency = 9.293 GHz, modulation amplitude = 10. Receiver gain = 5×10^4, Microwave power = 5 mW. (b–e) As (a) but plus stoichiometric addition of 4-nitro catechol, protocatechualdehyde, histazine and catechol respectively.

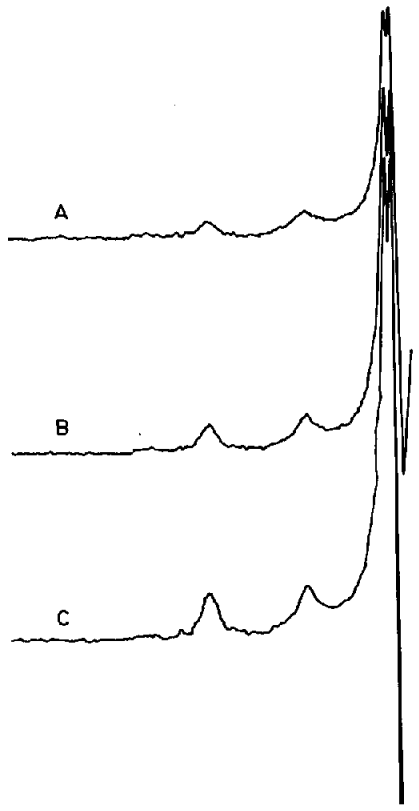

Fig.2. As fig.1b but at (a) 15°K; (b) 8.5°K; (c) 5.5°K.

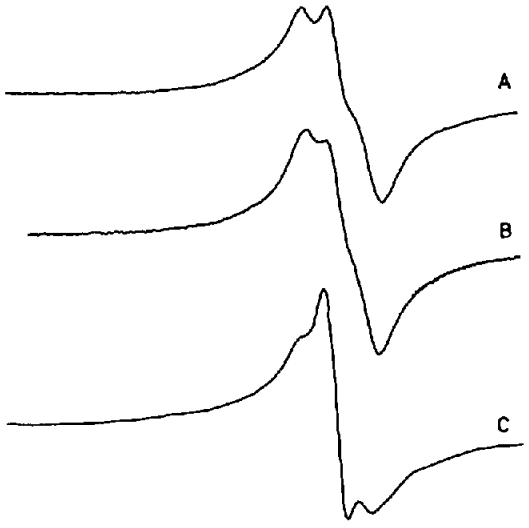

Fig.3. (a) As fig.1b H scale magnified × 10; (b) Nitrocatechol – 13-L-ROOH – Enzyme, ratio 6 : 1 : 1; (c) Nitrocatechol – 13-L-ROOH – Enzyme, ratio 1 : 6 : 1.

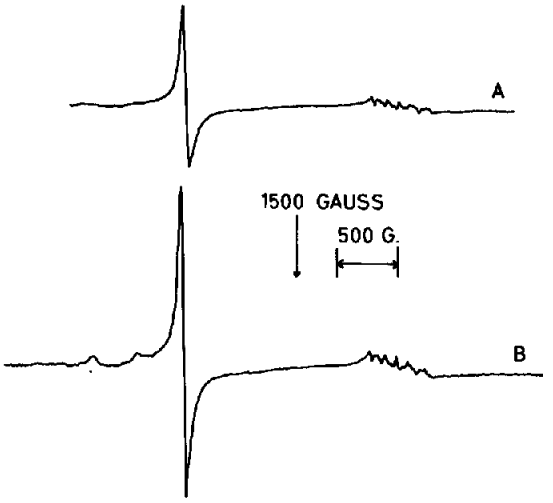

Fig.4. (a) As fig.1b but incubated for 48 h at 4°C; (b) As fig.1b but made anaerobic, followed by the addition of a 3 fold molar excess of the acetylenic analogue of linoleic acid.

activity and EPR signals will be published elsewhere) remove the splitting but fail to reduce the iron to the ferrous form as is observed in the absence of catechols [1]. Also it appears from the colour of the enzyme 4 NC complex that at pH 6.9 or 9.5 only one of the hydroxyl groups is dissociated.

4. Discussion

A number of catechols are shown to bind, apparently irreversibly, with the Fe III (yellow) form of lipoxygenase, but not with the native enzyme nor with the anaerobic native enzyme. It should be noted that only the latter form of the enzyme can safely be considered as ferrous. Although the aerobic native form is EPR silent it is possible that this is diamagnetic Fe III O_2^- complex [1]. It seems that the best inhibitors are those with electron withdrawing groups on the aromatic ring. The presence of a substituent α to the diol system may interfere with binding.

The inhibition experiments show that a ferric form of the enzyme arises during the normal oxidation of linoleic acid as was previously suggested [1]. It seems probable that the native enzyme is quite capable of producing 13-L-ROOH directly from linoleic acid without any prior activation.

The split signal seen around $g = 4.3$ on addition of 4 NC to the system must arise from an Fe III: 4 NC : 13-L-ROOH complex. The splitting of the signal at $g = 4.3$ cannot be explained by invoking two independent paramagnetic centres. The purity of the enzyme and the iron: enzyme ratio argue that there is only one such centre. It could be postulated that an equilibrium of two different rhombic ligand field structures are present around the iron. However, the reaction of 4 NC with the enzyme is virtually stoichiometric and apparently irreversible. Also it is known that an excess 13-L-ROOH must be added to the Fe III (yellow) form of the enzyme to give a complete transformation to the purple form. Figure [3] is in agreement with these observations. Only a small change is seen in the relative height of the splitting on increasing the 4 NC concentration beyond stoichiometry (fig.3b). A much greater change is seen on increasing the 13-L-ROOH concentrations but the splitting remains quite pronounced.

Thus although fig.3b may be a mixture of two complexes, both having 4 NC bound but only one binding 13-L-ROOH, fig.3c is likely to represent essentially one species. Consequently we favour the suggestion [9] that the splitting arises from a considerable directional anisotropy in the g-tensor of the middle Kramers doublet.

If, as is in all inorganic complexes, both catechol oxygens are involved in metal chelation, then, since it is clear that both the catechol and 13-L-ROOH can ligand to iron simultaneously it is necessary to postulate that there are only three ligands from the iron to the enzyme in this complex.

The Fe (III) enzyme (fig.1a) clearly has four main ligands because of the axial nature of the signal [1,3]. It is unlikely that hydroxyl groups can ligand to the iron, since on increasing the pH to 12 we fail to observe a low spin iron signal, as would be expected [10].

The nature of the ligands from the enzyme to the iron atom are still unknown. It is clear from the distortion of the $g = 6$ signal [1] that they are either chemically or spatially non-equivalent and this work indicates (unless the EPR spectrum can be ascribed to the rare seven liganded pentagonal bi-pyramidal structure of iron, which we feel is unlikely) that at least one of them can be displaced.

The EPR results with 4 NC show that 13-L-ROOH, linoleic acid and its acetylenic analogue all bind at one site on the enzyme and also suggest that the mode of inhibition of 4 NC cannot be ascribed to a simple stereochemical blocking of the active site. It thus seems likely that the inhibition is due to the electron withdrawing (i.e. oxidising) properties of the catechols. This could cause a shift in the redox potential of the iron preventing its reduction by linoleic acid.

Acknowledgments

We would like to thank Dr R. Wever and Dr B. F. van Gelder for the EPR facilities, Dr R. Aasa for useful discussions and suggestions for further work and Unilever, Vlaardingen, for enzyme purification facilities. This work was in part supported by funds from the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research.

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