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Substrate fatty acid activation in soybean lipoxygenase-1 catalysis

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The question of dioxygen coordination to iron in soybean lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) has been investigated by EPR spectroscopy. EPR data in combination with magnetic susceptibility measurements on both air-saturated and deoxygenated enzyme samples (Petersson, L., Slappendel, S. and Vliegenthart, J.F.G. (1985) Biochim. Biophys. Acta 828, 81–85) indicate that native lipoxygenase does not bind dioxygen to the iron atom. This implies that soybean lipoxygenase-1 functions solely by a mechanism of fatty acid substrate activation.

Introduction

In recent years, there have been many kinetic and spectroscopic studies of the reaction mechanism of the dioxygenation of linoleic acid ((9*Z*,12*Z*)-octadecadienoic acid) by soybean lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12; for a review, see Ref. 1). Our current hypothesis on the mechanism of action of this non-heme iron dioxygenase involves two catalytic cycles [2] (Scheme I). In the predominant cycle, the Fe(III) enzyme is reduced by linoleic acid [3], yielding a linoleyl radical [4]. The reduction step is stoichiometrically coupled with the rate-limiting hydrogen abstraction from C-11 in linoleic acid [5]. After reaction of ground state triplet dioxygen with the linoleyl radical, an electron is transferred from the Fe(II) in the enzyme to the intermediate peroxy radical. Thus the anion of the product, (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13*S*-HPOD), is formed and the Fe(III) enzyme is restored. This cycle is designated the Fe(III) cycle because the iron both enters and leaves the catalytic cycle in the Fe(III) state. An additional cycle (the Fe(II) cycle of Scheme I) has been suggested in order to explain the formation of hydroperoxylinoleic acid when the starting enzyme species contains Fe(II), and only linoleic acid is present. In this cycle an Fe(II) enzyme species with dioxygen coordinated is active (Scheme I) [2,7]. The product hydroperoxide formed in the Fe(II) cycle can then oxidize the Fe(II) enzyme to the Fe(III) enzyme and the catalysis proceeds through the Fe(III) cycle. Concerning the activity of the various enzyme species it has been reported that Fe(II) and Fe(III) enzyme are kinetically indistinguishable in steady-state experiments [8,9].

Dioxygenation of fatty acid would require activation by the enzyme of either dioxygen or fatty acid. Dioxygen activation seems to be possi-

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

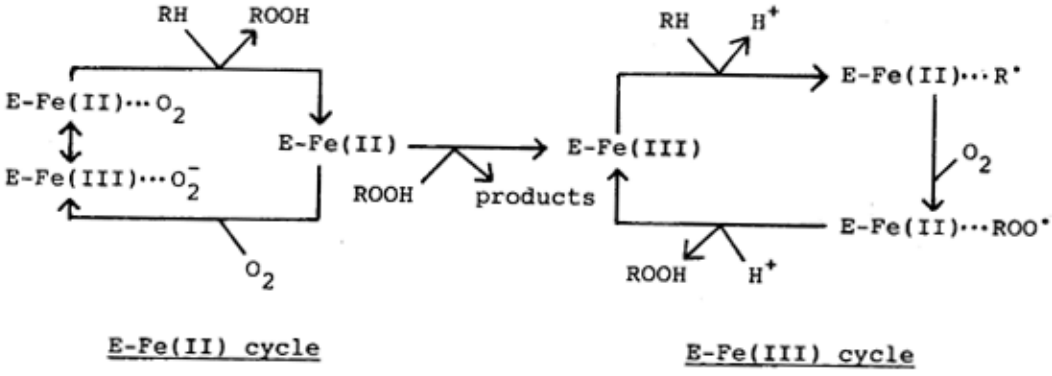
ble by interaction of dioxygen with the enzyme, particularly with the iron in the enzyme, as the primary step in the catalysis. Recently, magnetic susceptibility measurements have given some information on the possible dioxygen binding to iron [10]. No difference in Bohr magneton number was observed between air-saturated and deoxygenated Fe(II)-lipoxygenase. The deoxygenation procedure consisted of flushing with argon or nitrogen for 0.5 h. The results indicate that in the deoxygenated sample either the dioxygen is not bound to the iron, or it is bound so tightly to iron that it is not removed in this deoxygenation procedure. In order to differentiate between these possibilities, the anaerobic addition of linoleic acid to the nitrogen-flushed enzyme has been studied by EPR spectroscopy. One reagent that consumes any Fe(II)-bound dioxygen is the fatty acid substrate. If the enzyme is flushed with nitrogen and dioxygen is still present, anaerobic addition of linoleic acid should result in the formation of 13*S*-HPOD. Subsequently, iron will be oxidized, which can be monitored by the characteristic EPR signal [3]. If dioxygen is not present, 13*S*-HPOD will not be formed, and hence no EPR signal will be observed.

Materials and Methods

Llipoxygenase-1 was isolated from soybeans (*Glycine max* (L.) Merr. var. Williams) according to Slappendel [11], dialyzed against 0.1 M sodium borate buffer (pH 9.0) and diluted to 0.3 mM concentration. Linoleic acid (Lipid Supplies, St. Andrews University, St. Andrews, U.K.) was dissolved in 1 M ammonia in 252 mM concentration and then diluted in borate buffer to 0.3 mM. Both the protein and the substrate were flushed with nitrogen for 30 min.

For the anaerobic mixing of solutions of enzyme and substrate a piece of glassware with two compartments was used. It was connected to a valve which could be set either to nitrogen gas flow or to a rotary vacuum pump. The EPR tube was connected via a rubber tubing. The mixing cell was put under nitrogen atmosphere by repeated evacuation and flushing. 150 μl of the nitrogen-flushed solutions were then transferred to their compartments through the opening where the EPR tube was to be connected, while nitrogen gas was flowing. The solutions were rapidly frozen in their compartments in liquid nitrogen and the mixing cell was again repeatedly evacuated and flushed. Subsequently, its contents were thawed, carefully mixed, transferred into the connected EPR tube and frozen.

EPR spectra were recorded on a Varian-E9 spectrometer at 9 GHz and 15 K. For subsequent exposure to dioxygen, the contents of the EPR tube were thawed and transferred to a larger vessel, providing a large surface contact with the dioxygen gas flow in order to introduce dioxygen more effectively and to avoid consumption of 13*S*-HPOD in the anaerobic reaction [12]. After exposure for 30 min at 0°C the solution was transferred back into the EPR tube and frozen. Quantification of the EPR signal was carried out as described earlier [13,14].



Scheme I. Catalytic cycles of lipoxygenase. RH = linoleic acid; ROOH = linoleic hydroperoxide (13*S*-HPOD).

Results and Discussion

Native lipoxygenase was anaerobically mixed with linoleic acid. The EPR spectrum of the mixture is shown in Fig. 1A. Apart from a small feature at $g = 4.3$, no signal is observed. The spectrum in Fig. 1A suggests that the 1% iron EPR-visible at $g = 6$ in the native enzyme [13] has been reduced to Fe(II). The sample giving the spectrum in Fig. 1A was thawed, exposed to dioxygen and, subsequently frozen. The EPR spectrum of the oxygenated mixture is shown in Fig. 1B. Now a large $g = 6$ signal is observed, thus showing a 13*S*-HPOD-enzyme interaction [13]. This means that after introduction of dioxygen 13*S*-HPOD is formed and it demonstrates that the absence of dioxygen is the only reason that no product was formed. These experiments provide solid evidence that the method applied for the deoxygenation is effective. This also means that the possibility of a very tight binding of dioxygen can be ruled out as an interpretation of the magnetic susceptibility measurements [10]. It is therefore concluded that the native enzyme does not have dioxygen coordinated to iron.

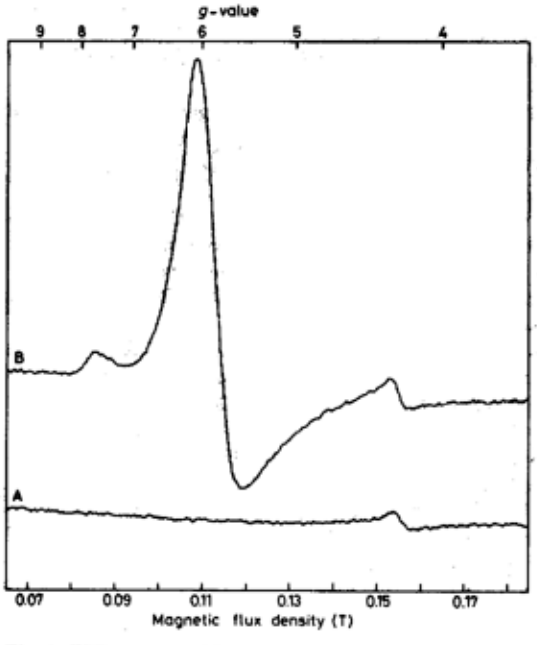


Fig. 1. EPR spectra of lipoxygenase-1. (A) Deoxygenated solutions of 150 µl 0.3 mM lipoxygenase-1 and 150 µl 0.3 mM linoleic acid were mixed anaerobically; (B) sample as described in (A), after introduction of dioxygen. Temperature, 15 K; receiver gain, $5 \cdot 10^3$; modulation amplitude, 2 mT; microwave power, 2 mW; microwave frequency, 9.22 GHz.

In parallel experiments the amount of EPR visible iron (at $g = 6$) has been established to be 40–60% of the total iron content. The same values were found in control experiments. For this purpose, enzyme and linoleic acid were treated as for the anaerobic experiment, but in this case air was admitted before mixing the solutions.

The interaction of lipoxygenase-1 with dioxygen and inhibitors of dioxygen binding has been described in the literature. Fluorescence studies [15] have demonstrated the existence of a low-fluorescent deoxygenated form of lipoxygenase-1. However, the slow recovery of the full fluorescence upon exposure to air is an indication that there are no groups in the enzyme exhibiting a large affinity for dioxygen. Carbon monoxide has no influence on the EPR spectrum of the Fe(III) enzyme [16]. In a study of the interaction of Fe(II) lipoxygenase with nitroxide [17], the EPR spectrum has been interpreted as stemming from high-spin Fe(III), which could arise from electron transfer from Fe(II) to nitroxide. Mainly on this basis, it has been proposed that iron in the native enzyme occurs in an iron-dioxygen complex, Fe(II)-O_2 ↔ Fe(III)-O_2^- [18,19]. However, our interpretation of the interaction of NO and lipoxygenase [17] has been questioned [20], and an explanation of the EPR spectrum in the sense of an antiferromagnetic coupling between high-spin Fe(II) and nitroxide is now favoured. This accounts for similar EPR signals of the complex of protocatechuate-4,5-dioxygenase with NO [21]. The isomer shift derived from the Mössbauer spectrum of the latter complex rather suggests a Fe(II) than a Fe(III) electronic configuration [22].

The lack of evidence for the coordination of dioxygen to iron makes it unlikely that a mechanism described by the Fe(II) cycle (Scheme I) exists. Another explanation for the start of the reaction of fully reduced lipoxygenase with substrate fatty acid has to be found. However, conclusive information in this respect is hard to obtain because of experimental difficulties concerning autoxidation of the substrate fatty acid and the occurrence of the trace amounts of protein-bound Fe(III).

The absence of dioxygen coordination to lipoxygenase excludes dioxygen activation by the enzyme. It seems that the iron atom in lipoxygenase is only involved in the activation of the substrate fatty acid by producing a fatty acid radical. In a recent survey on the enzymology of dioxygen [23], two general mechanisms by which metal-containing dioxygenases can act are distinguished. (1) One involves binding of dioxygen to the metal iron, in its low valence state, usually Fe(II), and activation by electron transfer from the metal ion to the bound dioxygen, as in indolamine-2,3-dioxygenase [24]. (2) The other involves the activation of the organic substrate. This is observed for protocatechuate-3,4-dioxygenase with iron present as Fe(III) [25]. In this respect, the mechanism of the catalysis of the dioxygenation by lipoxygenase is related to that of protocatechuate-3,4-dioxygenase because both enzymes act by organic substrate activation. However, in the case of protocatechuate-3,4-dioxygenase, iron remains Fe(III) throughout the catalytic cycle, whereas it changes valence in lipoxygenase.

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