Nitric oxide oxidises a ferrous mammalian lipoxygenase to a pre-activated ferric species

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Abstract Nitric oxide is known as an inhibitor of soybean lipoxygenase-1. Investigating the interaction of a mammalian 15-lipoxygenase with nitric oxide, we found that this enzyme is also inhibited reversibly when incubated with nitric oxide for a short time period (5 s) under anaerobic conditions. This inhibition may be due to the formation of a dissociable lipoxygenase-nitric oxide complex. With longer incubation periods the ferrous lipoxygenase is oxidised to a ferric form. This oxidation renders the enzyme more susceptible to peroxide activation, causing a time-dependent shortening of the kinetic lag phase.

Key words: X-ray absorption spectroscopy; Inflammation; Free radical; Eicosanoid

1. Introduction

Nitric oxide (NO) exhibits a variety of biologically relevant activities, such as smooth muscle relaxation, inhibition of cell proliferation, inhibition of platelet aggregation and down regulation of the expression of adhesion molecules [1–5]. In vitro studies on the interaction of NO with metalloproteins indicated its capability of complexing enzyme-bound ferrous ions [6,7]. The soybean lipoxygenase which contains 1 gatom non-heme iron per mol enzyme is reversibly inhibited by nitric oxide [6,8]. EPR studies indicated that this inhibition may be due to the formation of a ferrous lipoxygenase-NO complex [8,9]. For mammalian lipoxygenases which also contain 1 gatom iron/enzyme molecule, no experimental data are available on their interaction with NO. In the present study we investigated the reaction of the purified 15-lipoxygenase of rabbit reticulocytes with nitric oxide and found that NO is capable of oxidising the ferrous enzyme to a pre-activated ferric form.

2. Material and methods

2.1. Chemicals and preparations

Linoleic acid and sodium cholate were obtained from Serva (Heidelberg, Germany), NO gas from Linde (Munich, Germany). The rabbit reticulocyte 15-lipoxygenase was purified to electrophoretic homogeneity as described in [10]. The nitric oxide stock solution was prepared by repeated evacuation of 2 ml of 0.1 M phosphate buffer (pH 7.4) and re-equilibration with oxygen-free helium gas. Afterwards nitric oxide was bubbled through this solution for 20 min. Oxygraphic measurements indicated that the NO concentration of this solution varied between 1.5 and 3.0 mM in various sets of experiments. The solution of 15-lipoxygenase was partially anaerobised by gently blowing argon over it for several hours on ice. More vigorous methods such as securation or bubbling argon through the solution inactivated the enzyme. 2–5 μl of this enzyme solution were mixed with 5–100 μl of the NO stock under argon atmosphere and incubated for various time periods. Then 0.90–1.0 ml of aerobic phosphate buffer containing 250 μM linoleic acid and 0.2% sodium cholate was added and the lipoxygenase activity was measured spectrophotometrically recording the increase in absorbance at 235 nm.

2.2. Electron paramagnetic resonance spectroscopy (EPR)

EPR spectra were recorded with an ESR 300 spectrometer (ZWG, Berlin, Germany) at 4 K. For detection of the lipoxygenase-NO complex the lyophilised enzyme was flushed extensively with oxygen-free argon gas. Then NO gas was blown into the tube, the sample was quickly cooled to 4 K and EPR spectra were taken immediately afterwards. The control sample was handled identically with the exception that the enzyme was flushed with argon gas instead of NO. For long-term incubations 1 ml of the partially anaerobic enzyme solution (10 mg/ml) was mixed under an argon atmosphere with 2 ml of the NO stock solution, yielding a final NO concentration of about 1 mM. After 15 min of incubation the sample was frozen in liquid nitrogen, lyophilised and the EPR spectrum was taken at 4 K. In the control experiment the enzyme was treated in the same way with the exception that a saturated argon solution was added instead of the NO stock.

2.3. X-ray absorption near edge spectroscopy (XANES)

The enzyme samples for X-ray absorption spectroscopy were prepared in the same way as described for the EPR studies. The lyophilised enzyme was packed into a measuring cell (1 mm thickness) containing a Kapton-covered measuring window. X-ray absorption measurements were carried out with the EMBL (European Molecular Biology Laboratory) X-ray absorption spectrometer [11] at HASYLAB (c/o Deutsches Elektron Synchrotron, Hamburg). A Si(III) double crystal monochromator with an energy resolution of 1.9 eV at 7250 eV resulting in a $\Delta E/E = 2.6 \times 10^{-4}$ was used. The second monochromator crystal was detuned to 50% in order to reject harmonics of higher order. The monochromator angle was converted to an absolute energy scale by applying a calibration technique [11] resulting in an accuracy better than 0.2 eV. The X-ray absorption spectra were recorded by monitoring the X-ray fluorescence with a 13-element germanium solid-state detector. Series of 22–55 spectra were taken for each sample at 20 K in the range of 6900–7900 eV with steps of 0.3 eV in the near edge region. No evident damage of the protein sample occurred during exposure to the X-ray beam

3. Results

3.1. Kinetic studies

Lipoxygenases require small amounts of hydroperoxide for maximal enzymatic activity [12,13]. When hydroperoxide depleted substrates are used for the rabbit 15-lipoxygenase a kinetic lag period is observed [14]. We detected a kinetic lag period when the enzyme was pre-incubated for 5 s with nitric oxide under anaerobic conditions (Fig. 1). The maximal rates (inset) of linoleic acid oxygenation were somewhat lower with the NO-treated samples as compared with the untreated en-

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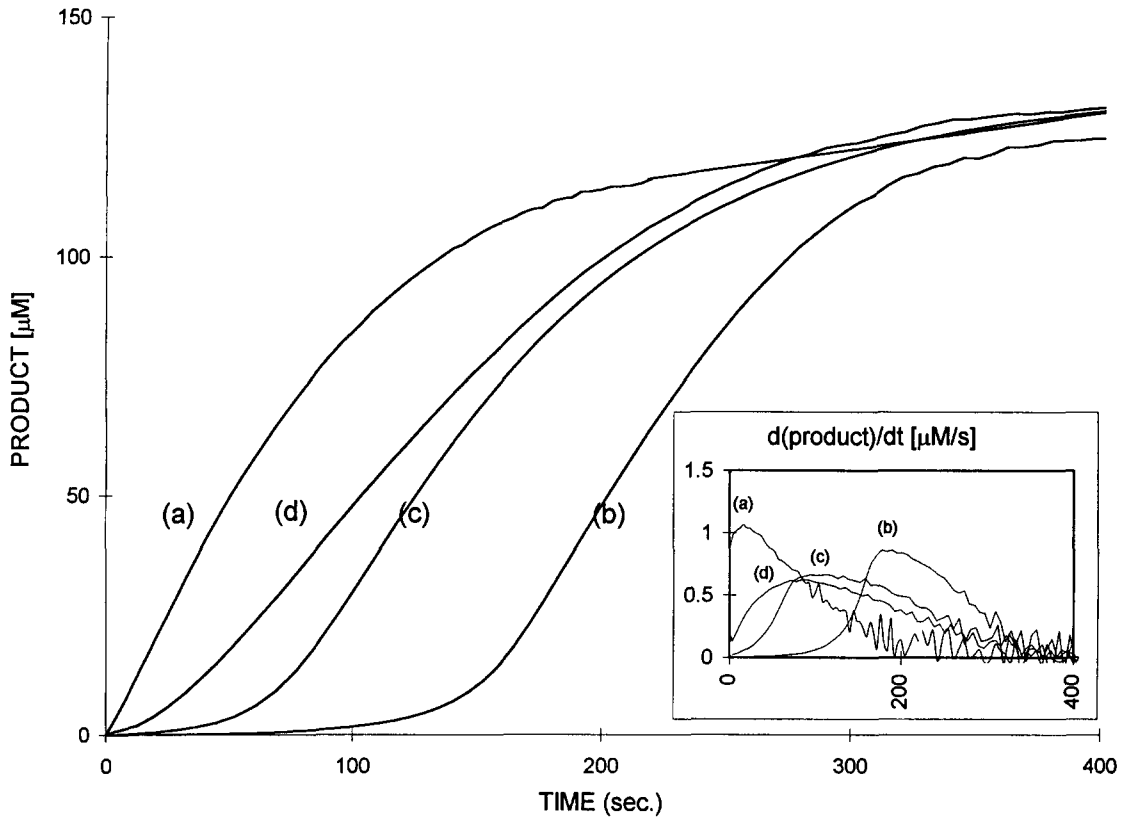

Fig. 1. Impact of NO on the kinetic lag phase of linoleic acid oxygenation. Pure rabbit reticulocyte 15-lipoxygenase (26 pmol) was incubated for various time periods in the absence and presence of NO (150 nmol) under anaerobic conditions; incubation volume, 100 μl. Afterwards, 0.9 ml of aerobic phosphate buffer containing 250 μM linoleic acid and 0.2% sodium cholate were added and the lipoxygenase activity was measured spectrophotometrically. (a) 5 s pre-incubation without NO (control), (b) 5 s pre-incubation with NO, (c) 60 s pre-incubation with NO, (d) 120 s pre-incubation with NO. Inset: First derivatives of the progress curves.

zyme. These data suggest that nitric oxide may reversibly interact with the rabbit 15-lipoxygenases and convert it to an enzyme species which is less susceptible to peroxide activation. A similar kinetic behaviour has been reported before for the soybean lipoxygenase-1 [6,8]. Furthermore, we found that the length of the lag period did depend on the NO concentration (not shown). Quite unexpectedly, we observed a shortening of the lag phase for longer pre-incubation periods (Fig. 1).

3.2. Spectroscopic studies

The mechanistic reasons for the appearance of the kinetic lag phase during short-term interaction of the lipoxygenase with NO may involve the formation of a dissociable complex between the ferrous lipoxygenase and NO. In order to obtain direct evidence for such a complex, EPR studies were carried out. The EPR spectrum of the native rabbit 15-lipoxygenase (Fig. 2A, trace a) is characterised by a weak signal at $g' = 4.3$ and a stronger one in the region of $2 < g' < 2.5$. These signals were consistently observed with various enzyme preparations ($n = 4$). The signal at $g' = 4.3$ which may be due to the presence of ferric iron co-ordinated in the 15-lipoxygenase molecule was strongly augmented when the enzyme was treated with hydroperoxy linoleic acid (not shown). The structural reasons for the signal in the $2 < g' < 2.5$ region are unclear. When the 15-lipoxygenase was reacted with NO for a short time period (Fig. 2A, trace b) a strong signal at $g' = 4.1$ was detected. By computer simulation this signal was assigned to a ferrous iron-NO complex with a spin state s' of 3/2 and a zero field splitting D of about 15 cm^{-1}.

The time-dependent shortening of the kinetic lag phase (Fig. 1) suggested that nitric oxide may convert the lipoxygenase to an enzyme species which is more susceptible to peroxide activation. Activation of lipoxygenases by hydroperoxy fatty acids is a two-step process involving the oxidation of the ferrous lipoxygenase and the subsequent formation of a purple-coloured ferric lipoxygenase-hydroperoxide complex [15,16]. If oxidation of the ferrous lipoxygenase were to take place during the lipoxygenase-NO interaction, the time-dependent shortening of the kinetic lag phase would become plausible. To test this hypothesis, the 15-lipoxygenase was incubated with NO for 15 min and its EPR spectrum was taken. The spectrum of the control sample (Fig. 2B, trace c) which was not treated with nitric oxide but was taken through the same experimental protocol showed a weak signal at $g = 4.3$ indicating that a small percentage of the enzyme was present in its ferric form. Moreover, a signal of unknown structural origin at $g' = 1.98$ was observed. NO treatment resulted in an EPR spectrum (Fig. 2B, trace d) which was characterised by at least 4 different paramagnetic species: (i) axial ferric species contributing to the signals at $g' = 6.03$ and 2.00; (ii) rhombically distorted ferric species with contribution to the signals at $g' = 10.43$, 4.30 and 2.00; (iii) less rhombically distorted and/or coupled ferric species with contributions to the signal at $g' = 3.03$; and (iv) unknown oxygen radicals which may give rise to the signal at $g' = 1.98$. These data

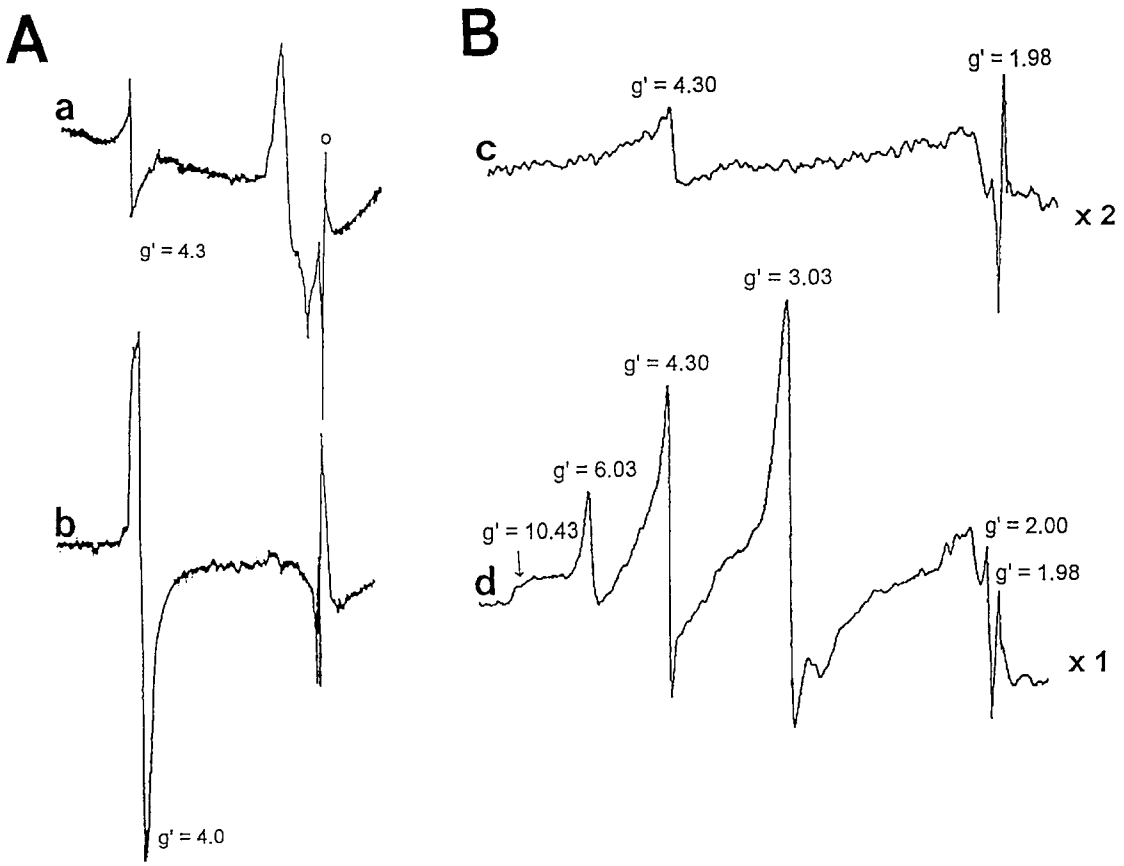

Fig. 2. EPR spectra of the rabbit 15-lipoxygenase after its interaction with NO. (A) Native rabbit 15-lipoxygenase (10 mg) was lyophilised from a volatile NH_4HCO_3 buffer (pH 7.6), packed into a 3 mm quartz tube and was flushed extensively with argon to remove oxygen. After taking the EPR spectrum at 4 K (trace a), nitric oxide gas was blown into the tube and the EPR spectrum was taken again (trace b). The signal of an internal standard is marked by a circle. (B) To 1 ml of a partially anaerobised 15-lipoxygenase solution (10 mg/ml) 2.0 ml of the nitric oxide stock solution (3 mM) were added. After 15 min of incubation the sample was frozen in liquid nitrogen, lyophilised and the EPR spectrum was taken at 4 K (trace d). In the control experiments (trace c) the enzyme was treated in the same way with the exception that a saturated argon solution was added instead of the NO stock. Note that the sensitivity of the control trace was twice that of the NO-treated sample.

suggest that nitric oxide treatment of mammalian lipoxygenases may lead to oxidation of the ferrous enzyme species.

Independent evidence for such oxidation was provided by X-ray absorption spectroscopy in the iron near edge region (XANES). It can be seen from Fig. 3 that the edge position for the native lipoxygenase was found at 7120.5 eV, a value characteristic for ferrous ions. A similar spectrum has been reported recently for this enzyme [17,18] and for the soybean lipoxygenase [18,19]. However, after treatment with nitric oxide the edge position was shifted to 7123.5 eV indicating the presence of ferric ions.

4. Discussion

Both NO [2,20] and lipoxygenase products such as leukotrienes and hydroxy fatty acids [21–23] are messenger molecules which exhibit a variety of biologically relevant properties. So far no direct interaction of both pathways in mammalian cells has been described. The data presented here indicate that nitric oxide is capable of interacting with a mammalian lipoxygenase and thus, may be regarded as a physiological modulator of the cellular lipoxygenase pathways. Earlier studies on the soybean lipoxygenase characterised NO as reversible inhibitor of plant lipoxygenases [8,9]. The data presented here indicate that a mammalian lipoxygenase is also inhibited when co-incubated for a short time period with NO. In addition and quite unexpectedly, we found that NO is capable of oxidising the enzyme-bound ferrous non-heme iron when incubated with the lipoxygenase for longer time periods. This oxidation may be regarded as preactivation of the enzyme and is reflected by a shortening of the kinetic lag phase. These data suggest that NO may act as reversible inhibitor as well as activator of mammalian lipoxygenases depending on the duration of their interaction.

Although the mechanism of the NO-induced oxidation of the rabbit reticulocyte 15-lipoxygenase has not been studied in detail, it may proceed in a similar way to NO-induced methemoglobin formation [24]. Under our experimental conditions, the oxidation took place in the anaerobic pre-incubation mixture in which only the lipoxygenase and NO were present. Thus, an indirect effect of nitric oxide derivatives, such as peroxynitrile, appears unlikely. It should, however, be stressed that despite our efforts to exclude oxygen from the pre-incubation mixture to prevent massive NO autoxidation, complete anaerobiosis was certainly not achieved. Thus, NO-autoxidation intermediates may be involved in NO-induced lipoxygenase oxidation. In additional experiments, we excluded the possibility that the oxidation of the lipoxygenase is a

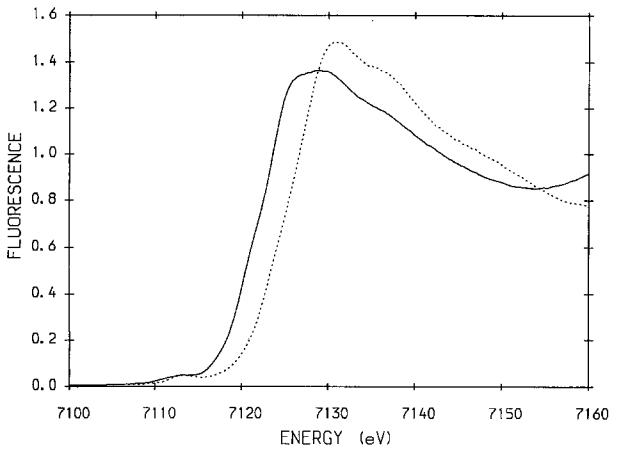

Fig. 3. X-ray absorption spectra in the near edge region of the rabbit 15-lipoxygenase. The preparation of the lipoxygenase samples was carried out as described in the legend to Fig. 2. XANES spectra were taken as described in Section 2. Solid line, native lipoxygenase (no NO treatment); dotted line, NO treated lipoxygenase.

lyophilisation artefact. When the lyophilised ferrous lipoxygenase was flushed with nitric oxide gas for 1 h and X-ray absorption spectra were taken subsequently a shift in the iron edge was observed, indicating that the oxidation the enzyme-bound iron proceeds during lipoxygenase-NO interaction.

At this time it remains to be investigated whether the effects reported here are of cell physiological relevance. Preliminary kinetic studies on the lipoxygenase-nitric oxide interaction indicated a K_d for the formation of the lipoxygenase-NO complex of about 2.5 μM. Although no reliable data on intracellular NO concentrations are available, measurements of its extracellular steady-state concentration suggest [2,25] that μM concentration may well be reached at the site of NO synthesis.

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