INVOLVEMENT OF MYELOPEROXIDASE IN THE METABOLIC ACTIVATION OF PHAGOCYTES: EPR STUDIES

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1. Introduction

Myeloperoxidase, the peroxidase of polymorphonuclear leucocytes, is associated with the azurophilic granules of these cells [1,2]. The principal function presently attributed to this enzyme is that of providing one component of the microbicidal system myeloperoxidase–H_2O_2–halide (for review see refs [3] and [4]). In addition, myeloperoxidase has been proposed to play a role in the increased oxygen consumption and hydrogen peroxide production in phagocytosing leucocytes [5]. This putative role might be performed in two ways, (1) through the oxidase activity of myeloperoxidase on an available substrate (most likely NADPH) which leads to H_2O_2 production via O_2^- [6] and (2) by release of O_2^- from oxyperoxidase, the oxygen adduct of the ferrous myeloperoxidase, the radical being thereafter dismutated to H_2O_2 and O_2 [7]. The relevance of these two mechanisms in vivo is still to be clarified. Wever et al. have shown by electron paramagnetic resonance (EPR) that the high spin heme signal of ferric myeloperoxidase is slowly converted during phagocytosis from a rhombic to an axial signal [8]. Thus the EPR signal of myeloperoxidase may represent a useful tool to study the involvement of this enzyme in the oxidative change occurring in leucocytes during phagocytosis.

This problem has been approached experimentally in the present study by recording the EPR spectrum of:

(1) Phagocytosing cells exposed to CN^- which does not inhibit either the oxidative burst or the ingestion of particles [9]

(2) Phagocytosing cells under anaerobiosis

(3) Granules isolated from resting and phagocytosing cells to test the stability of the signal during the isolation of granules and

(4) Partially purified myeloperoxidase as a reference.

2. Materials and methods

2.1. *Leucocytes*

Guinea pig polymorphonuclear leucocytes (PMNL) were obtained from peritoneal exudates induced by a 50 ml injection of 1% sterile sodium caseinate in isotonic saline. The exudates were collected 14 h later and the cells were harvested by centrifugation at 250 × *g* for 5 min. Contaminating erythrocytes were lysed by suspending the pelleted cells in hypotonic saline (0.2% NaCl) for 30–60 sec. After restoring the tonicity with an appropriate volume of 1.2% NaCl, the cells were centrifuged and resuspended in Krebs Ringer Phosphate buffer pH 7.4 without Ca^{2+} (KRP). The percentage of PMNL in the preparations used was never less than 85%.

2.2. *Leucocyte incubation*

2 ml of cell suspension (2×10^8 cells/ml) in KRP, were incubated with heat-killed opsonized *B. mycoides* (100 bacteria/cell) at 37°C for 15 min or 30 min in the upper compartment of a Thunberg type EPR tube. Resting cells were incubated in the same conditions but

without bacteria. At the end of the incubation the EPR tube was centrifuged for 15 min at low speed, to obtain a final volume of 0.5 ml with 8×10^8 cells/ml.

2.3. *Preparation of granules*

Leucocytes (4×10^7 cells/ml) were incubated in KRP, with heat-killed opsonized *B. mycoides* (100 bacteria/cell) for 15 min at 37°C in regular plastic tubes. Resting leucocytes were incubated without bacteria. A mixture of 95% CO_2 and 5% O_2 was bubbled in the tube throughout the incubation time. At the end of the incubation the mixture was diluted out with cold KRP. The cells were sedimented at $150 \times g$ for 10 min, which left most of the bacteria in suspension then resuspended in a small volume of 0.34 M sucrose (30×10^7 cells/ml). This suspension was homogenized in a glass homogenizer equipped with a Teflon pestle driven by a motor. The number of disrupted cells was checked by light microscopic examination. After dilution with 0.34 M sucrose, the homogenate was centrifuged at $250 \times g$ for 5 min to remove nuclei, cell debris and unbroken cells. The supernatant was collected and centrifuged at 20 000 $\times g$ for 15 min to sediment the granules. The pellet was resuspended in 0.34 M sucrose, so that the suspension contained in 1 ml roughly the number of granules deriving from 10^9 cells.

2.4. *Myeloperoxidase*

Myeloperoxidase was purified from isolated granules as starting material. The granules isolated from 52×10^9 cells were extracted with 20 vol. 0.02 M acetate buffer, pH 4.7, containing 0.025% digitonin and 0.35 M Na_2SO_4. The supernatant was dialysed against 0.02 M acetate buffer, pH 4.7, containing 0.2 M NaCl then passed through a C-50 CM–Sephadex column equilibrated with the same buffer. A linear concentration gradient of NaCl (0.2–1.2 M) was used for the elution. The fractions having myeloperoxidase activity were pooled and concentrated. The enzyme activity was measured with the *o*-dianisidine method [10]. The enzyme concentration was estimated from the absorption at 430 nm [8], assuming that the absorption coefficient was the same as the human enzyme.

2.5. *EPR spectra*

X-band EPR spectra were recorded with a V-4502 Varian spectrometer equipped with an Air Products and Chemicals LT-3-110 liquid transfer Cryo-Typ refrigerator with automatic temperature controller.

3. Results

The EPR spectrum of isolated guinea pig ferrimyeloperoxidase shows (fig.1. curve a) a strongly rhombic

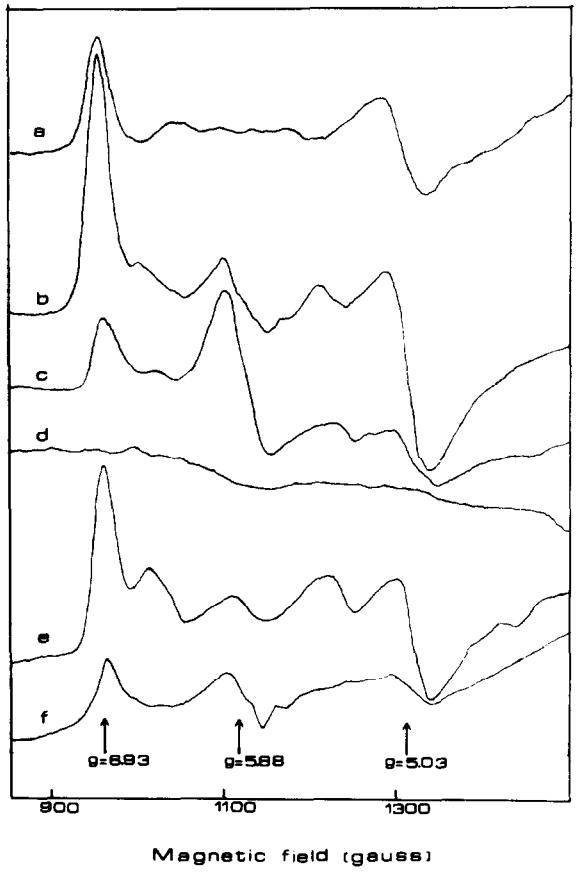

Fig.1. EPR spectra of isolated myeloperoxidase and of resting and phagocytosing granulocytes. (a) Purified myeloperoxidase, approximately 100 μM, in 0.02 M sodium acetate, pH 4.7, containing 1 M NaCl. (b) Resting PMNL incubated for 30 min at 37°C. (c) (b) After 30 min incubation with heat-killed bacteria at 37°C. (d) (b) or (c) after addition of 1 mM CN^-. The same result was obtained when CN^- was added at the beginning of the incubation, (e) as (b) but in anaerobiosis, (f) as (c) but in anaerobiosis. EPR conditions: frequency 9.24 Ghz, microwave power 6 mW, modulation amplitude 10 G, temperature 7°K.

high spin heme iron signal. The *g*-values are very close to those reported by Wever et al. [8] for the human enzyme. The same signal is the predominant species in the EPR spectrum of guinea pig resting granulocytes (curve b) found by Wever et al. [8] for the human cells. Other high spin heme iron signals are present, and an axial signal with a *g*-value near 6 is clearly evident.

Induction of phagocytosis leads to an increase of the axial signal and at the same time to a decrease of the major rhombic species (curve c). The conversion of the rhombic signal into the axial one is a slow process, since the intensity of the latter increases very clearly be increasing the incubation time with bacteria (the signal is much more evident after 30 min incubation than after 15 min incubation). Addition of cyanide either at the end or at the beginning of the incubation period leads to the disappearance of the EPR signal both in the resting and in the stimulated leucocytes (curve d). Incubation under anaerobic conditions produces obvious changes in the overall intensity of the spectrum, but does not seem to modify the type of response of the phagocytes (curves e and f).

The isolated granules show a qualitatively similar behavior to that of intact cells. (Fig.2). It should, however, be pointed out that in the granules isolated from the resting cells, the axial signal has an intensity comparable to that of the rhombic one (curve a). The detection of the axial signal also in the granules isolated from resting cells is difficult to be interpreted, since there is no means to rule out the possibility that the resting cells contained a small percentage of cells stimulated, say, by phagocytosis of fibrin clots in the peritoneal exudate.

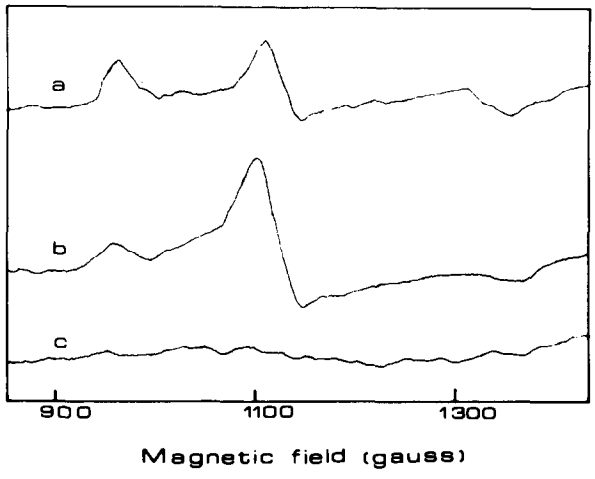

Fig.2. EPR spectra of isolated granules. (a) Granules isolated from resting cells. (b) Granules isolated from phagocytosing cells. (c) (a) or (b) after addition of 1 mM CN^-. EPR conditions as in fig.1.

Attempts to induce the species characterized by the axial signal in the isolated enzyme were unsuccessful. The EPR spectrum of the enzyme is insensitive to pH changes between 4.0 and 8.4, as well as to addition of halides and a slight excess of H_2O_2. Addition of more than ten-fold excesses of H_2O_2 denatures the enzyme at acid pH, while leading to reversible disappearance of the EPR signal at alkaline pH. The latter phenomenon is likely to reflect the formation of oxymyeloperoxidase [11].

4. Discussion

The results presented here are in agreement with the findings reported by Wever et al. [8] for human granulocytes as far as they overlap. The EPR signal of myeloperoxidase is easily observed in intact granulocytes as a rhombic high spin heme iron signal, together with a signal of axial line shape with $g \simeq 6$. The latter signal slowly increases with time when the cells are induced to phagocytose.

Our results also show that these signals can be observed in the isolated intracellular granules, and that the granules from phagocytosing cells show far more axial signal than those from resting cells. It can be safely concluded from our results that the change in the EPR spectrum of myeloperoxidase cannot be uniquely correlated with the respiratory burst occurring upon phagocytosis. This is supported by the following considerations:

(1) The appearance of the axial signal is a slow process, occurring several minutes after cell stimulation, while the respiratory burst occurs a few seconds after stimulation.

(2) The axial signal is abolished by cyanide at concentrations which have no effect on the respiratory burst [9].

(3) The rhombic → axial conversion occurs also in cell phagocytosing under anaerobic conditions.

The axial signal therefore appears to reflect some modification of myeloperoxidase which is related to, but possibly not uniquely determined by, the phagocytic act. The origin and the nature of the observed spectral changes are still unknown also in view of the fact that the spectrum of the guinea pig enzyme is insensitive to changes in pH and to the presence of molecules such as H_2O_2 and halides, which are supposed to play a role in the bacterial killing. In particular, we were unsuccessful in obtaining the axial signal from the isolated enzyme.

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