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Interaction of human myeloperoxidase with nitrite*

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EPR (electron paramagnetic resonance) and optical spectroscopy show that human neutrophil myeloperoxidase is converted from ferric high-spin to low-spin by the addition of nitrite. The Soret peak shifts from 429 to 447 nm and new peaks appear in the visible region at 573 and 627 nm; the EPR g-values change from 6.84, 5.02, 1.95 to 2.55, 2.31, 1.82. Small differences are seen in the EPR (but, not optical) spectra of myeloperoxidase isoenzyme I compared to isoenzymes II and III. The reaction with nitrite is detectable by EPR in intact neutrophils and is thus a possible in vivo monitor of NO/nitrite production by these cells.

Myeloperoxidase; Isoenzyme; Neutrophil; Electron paramagnetic resonance; Nitrite; Nitric oxide

1. INTRODUCTION

Myeloperoxidase (donor H_2O_2 oxidoreductase, EC 1.11.1.7) is an abundant haem peroxidase in human neutrophil granules and consists of a dimer of two heavy and two light chains with two haem prosthetic groups [1]. In activated neutrophils MPO reacts with hydrogen peroxide and superoxide radical produced by the neutrophil respiratory burst to form hypochlorous acid [2] which is important for microbial killing [3]. The enzyme exists as three major isoenzymes [4] although the basis for the heterogeneity is not clear [5–7].

Neutrophils and macrophages release nitric oxide formed from the guanido nitrogen of L-arginine and molecular oxygen by the NADPH-dependent cytosolic enzyme nitric oxide synthase [8]. The role of nitric oxide in macrophages and possibly neutrophils includes the killing of microbes [9] and tumour cells [10]. Following release the nitric oxide reacts further in vivo to form nitrite and nitrate [11].

As both nitric oxide and nitrite are reactive with iron-containing proteins we investigated their reactions with human neutrophil myeloperoxidase. Recently lactoperoxidase, another haem peroxidase with similarities to myeloperoxidase [12], has been shown to react with nitrite [13] and a nitrosyl derivative of myeloperoxidase has been described [14]. Such interactions might occur in vivo – nitrosyl derivatives of mitochondrial iron-sulphur centres have been detected in murine target cell lines exposed to activated macrophages [15].

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2. MATERIALS AND METHODS

Myeloperoxidase was purified according to Matheson et al. [16] from neutrophil primary granules separated by discontinuous density gradient centrifugation as previously [17] except that dithiothreitol was omitted. Myeloperoxidase isoenzymes were prepared separately from neutrophils according to Miyasaki et al. [4] except that the initial gel filtration step was replaced by ultrafiltration against a 100,000 MW cut-off filter (Amicon YM100, Amicon Corp., Danvers, MA, USA). Normal human neutrophils were isolated from buffy coat residues by dextran sedimentation and Ficoll–Hypaque centrifugation and suspended in 0.9% NaCl. All optical spectra were recorded at 25°C on a Perkin Elmer Lambda 2 spectrophotometer. Continuous-wave EPR measurements were recorded on a Bruker ESP300 spectrometer fitted with a TE103 rectangular cavity, a Hewlett Packard microwave frequency counter 5350B and an Oxford instruments liquid helium flow cryostat ESR900. Spectra were baseline corrected by subtraction of a cavity spectrum or water/buffer under identical conditions. Conditions of EPR spectra: temperature 30 K, modulation frequency 100 kHz, modulation amplitude 1 mT, microwave frequency 9.35 gHz, microwave power 20 mV, scan time 2.4 $mT \cdot s^{-1}$; spectra are averages of 3 scans. Owing to a lower signal:noise ratio the neutrophil spectra were recorded at a four-fold higher gain than those for the pure enzyme.

3. RESULTS

As purified, both iron centres of myeloperoxidase are high spin [18,19] with a Soret peak at 429 nm and visible peaks at 570 and 689 nm (Fig. 1). The addition of nitrite induces a red-shift of the Soret peak to 447 nm and new peaks in the visible region at 573 and 627 nm. Clear isosbestic points are seen at 441.5 nm and 650 nm; at 596 nm the isosbestic point is less well-defined. Nitrite induces similar shifts in the lactoperoxidase spectrum [13], although the absolute values of the peaks are blue-

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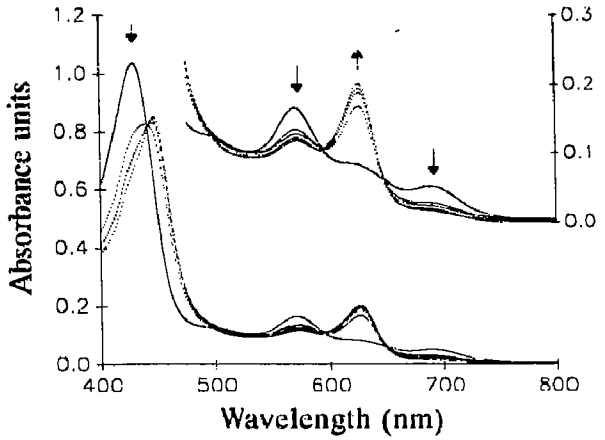

Fig. 1. Changes in optical spectra following nitrite addition to myeloperoxidase. Sequential 5 mM nitrite additions to 5.6 μM myeloperoxidase (mixed isoenzymes) in 10 mM sodium phosphate, pH 7.4. Solid line is spectrum prior to nitrite addition. Each spectrum was allowed to develop fully prior to the subsequent addition. Arrows show the direction of absorbance shift with the addition of nitrite.

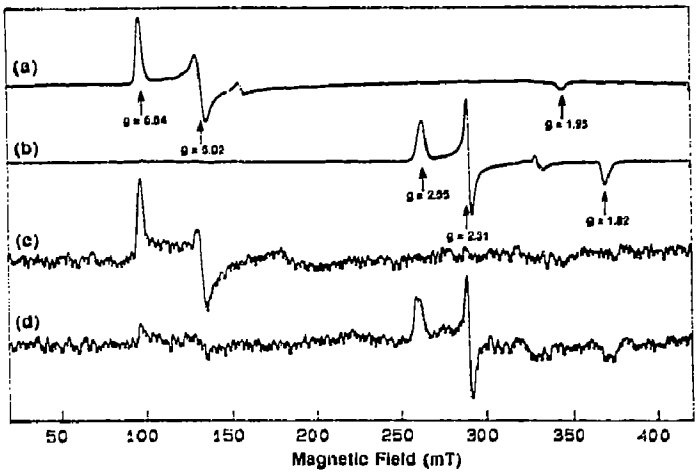

Fig. 2. Changes in EPR spectra following nitrite addition to myeloperoxidase. 95 μM myeloperoxidase in 10 mM Na-HEPES, 100 mM NaCl, pH 7.0 (a); 95 μM myeloperoxidase + 20 mM nitrite (b); neutrophils as prepared (c); neutrophils plus 20 mM nitrite (d). For EPR conditions see section 2.

shifted by 20 nm compared to myeloperoxidase due to the differences in the nature of the prosthetic group. Using the shifts in either the Soret or visible regions the value for the dissociation constant for nitrite binding to myeloperoxidase was estimated to be 1–2 mM, showing a tighter binding than is observed for lactoperoxidase [13].

The red-shift in the Soret spectrum suggests that the addition of nitrite induces a high–low spin shift in the iron centre. This was confirmed by the use of EPR spectroscopy (Fig. 2). The haem in our preparation is homogeneous high-spin with g-values of 6.84, 5.02 and 1.95. The addition of chloride has little effect on this spectrum (Fig. 2a). The addition of nitrite converts the enzyme to a low-spin form with g-values at 2.55, 2.31 and 1.82 (Fig. 2b). Quantitation of this low-spin form shows essentially 100% of the high-spin iron is converted to the low-spin form. Bolscher and Wever [14] were able to produce the same signal upon addition of NO to the oxidised or reduced enzyme (especially when dithionite was used as a reductant). We were unable to produce the low-spin signal upon the anaerobic addition of NO to the oxidized enzyme. However, we were able to observe this signal upon addition of excess nitrite to the dithionite-reduced enzyme. The g-values of the low-spin form are similar to that seen upon addition of nitrite [13] to lactoperoxidase (2.53, 2.32 and 1.81). In agreement with our findings, Lukat et al. [13] found that addition of NO to ferric lactoperoxidase did not generate this low spin signal, but the ferrous–NO complex was slowly converted into the low-spin complex as oxygen diffused into the system.

The low-spin complex can also be prepared in vivo. As prepared, a significant fraction of myeloperoxidase in neutrophils is high-spin ferric [20] and therefore EPR detectable (Fig. 2c). The absence of signals at g = 6.0 shows that there is little contamination with eosinophils [21]. The addition of 20 mM nitrite to these neutrophils quantitatively converts this fraction to the low-spin ferric form (Fig. 2d). Similar low-spin haemoglobin complexes can be seen following the addition of nitrite to erythrocytes [22] although the reaction is not as homogeneous as that seen here.

There are three major isoenzymes of myeloperoxidase. Table I shows the g-values for the purified chloride and nitrite complexes of these isoenzymes. In all three complexes there is a small, but significant, difference in the g-values of isoenzyme I compared to isoenzymes II and III. Isoenzyme I has a smaller rhombic distortion in its high-spin EPR signals representing small differences in the values of the zero-field splitting parameters (D/E); in the low-spin complex there is slightly greater rhombic distortion in isoenzyme I, presumably due to small differences in the spin-orbit coupling constant. No differences were seen in the optical spectra of the myeloperoxidase isoenzymes prepared under the same conditions as in Table I.

4. DISCUSSION

These studies further strengthen the spectroscopic similarities between myeloperoxidase and lactoperoxidase. Both form a low-spin complex upon addition of nitrite to the oxidized form. The same complex appears to be formed upon oxidation of the ferrous–NO complex. The fact that nitrite is a strong field ligand suggests that, as proposed by Lukat et al. [13] this complex is Fe^{3+}/NO_2^-. However, the same EPR signals are seen as a minor low-spin component of some myeloperoxidase [23–25] and spleen green haemoprotein preparations

Table 1

Myeloperoxidase complexes formed by addition of 100 mM sodium chloride or 20 mM sodium nitrite to purified enzyme or neutrophils

Isoenzyme	g-values		
	As prepared	+Chloride	+Nitrite
I	6.815, 5.05, 1.955	6.82, 5.035, 1.953	2.563, 2.304, 1.812
II	6.84, 5.02, 1.950	6.85, 5.01, 1.950	2.547, 2.308, 1.816
III	6.84, 5.02, 1.950	6.84, 5.01, 1.950	2.545, 2.309, 1.816
Neutrophil	6.84, 5.00, ND	ND	2.56, 2.30, 1.80

In agreement with Wever and Bakkenist [18] we observe only small changes to the high-spin EPR signals upon addition of chloride to myeloperoxidase. Larger changes appear to be observable at pH 4.0 [23]. Other conditions as per Fig. 1. ND = not determined. g-values of neutrophils are less accurate due to lower signal:noise ratios.

[26]. Either nitrite is not a haem ligand in these species or these preparations have some nitrite-bound enzyme as purified. The latter appears unlikely given the rather low affinity for nitrite reported for the putative nitrite complex in both myeloperoxidase (this paper) and lactoperoxidase [13]. The resolution to this problem awaits a direct measurement of nitrite binding to the iron moiety.

The preference for myeloperoxidase to form a low-spin iron complex in the presence of dithionite and NO [14] and dithionite and nitrite (this paper) suggests that, as for lactoperoxidase [27], the enzyme may use dithionite and/or its oxidation products as a substrate. This is especially likely if any oxygen is present as this will react with dithionite to produce H_2O_2. In the absence of oxygen it is difficult to understand the formation of a low-spin haem complex, following dithionite and NO addition, unless there are additional side-reactions involving NO itself.

The EPR spectra of myeloperoxidase isoenzyme I is distinct from isoenzymes II and III. The same small difference in the chloride complex EPR spectra was reported previously, but not considered significant [7]. However, our confirmation of this observation (and similar results with the enzyme as purified and with nitrite) confirms a small, but significant, difference in the EPR spectra of isoenzyme I. The differences between the spectra of isoenzyme I and isoenzymes II/III are very unlikely to be due to a change in the ligand at the active site; however, they may be responsible for some of the differences seen in the high-spin EPR spectra as prepared by different authors [7,18,23-25]. It is interesting that the isoenzyme with distinct substrate reactivity [7] is also that with distinct EPR spectra, although whether these two observations are linked to the same physical difference remains to be determined.

The ability of myeloperoxidase in neutrophils to react with nitrite suggests that myeloperoxidase could be used as a direct monitor of NO formation in vivo, especially as the superoxide present in activated neutrophils would be expected to convert NO to nitrite. Experiments are currently under way to test this hypothesis.

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