

Resonance Raman Microspectroscopy of Myeloperoxidase and Cytochrome b_{558} in Human Neutrophilic Granulocytes

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ABSTRACT With (resonance) Raman microspectroscopy, it is possible to investigate the chemical constitution of a very small volume (0.5 fl) in a living cell. We have measured resonance Raman spectra in the cytoplasm of living normal, myeloperoxidase (MPO)-deficient, and cytochrome b_{558} -deficient neutrophils and in isolated specific and azurophilic granule fractions, using an excitation wavelength of 413.1 nm. Similar experiments were performed after reduction of the redox centers by the addition of sodium dithionite. The specific and azurophilic granules in both redox states appeared to have clearly distinguishable Raman spectra when exciting at a wavelength of 413.1 nm. The azurophilic granules and the cytochrome b_{558} -deficient neutrophils showed Raman spectra similar to that of the isolated MPO. The spectra of the specific granules and the MPO-deficient neutrophils corresponded very well to published cytochrome b_{558} spectra. The resonance Raman spectrum of the cytoplasmic region of normal neutrophilic granulocytes could be fitted with a combination of the spectra of the specific and azurophilic granules, which shows that the Raman signal of neutrophilic granulocytes mainly originates from MPO and cytochrome b_{558} , at an excitation wavelength of 413.1 nm.

INTRODUCTION

Neutrophilic granulocytes play an important role in the human immune system. It is their task to migrate to sites in the body where an infection has occurred and to ingest and destroy the bacteria responsible for the infection. Neutrophils contain two heme proteins that are of importance in this process (Borregaard, 1988): cytochrome b_{558} , which is localized in the membranes of the specific or secondary granules and the secretory granules, and myeloperoxidase (MPO), which is concentrated in the azurophilic or primary granules. Neutrophils ingest invading microbes in a so-called phagosome. Both the azurophilic and specific granules fuse with this phagosome. MPO is released into the phagosome, and the cytochrome b_{558} is translocated to the phagosome membrane. Cytochrome b_{558} is the central protein in the NADPH oxidase leading to the formation of superoxide anions, which dismutate to hydrogen peroxide. MPO catalyzes the oxidation of chloride to the bactericidal agent hypochlorous acid by hydrogen peroxide.

MPO consists of two monomers that are covalently linked to one another by a single disulfide bridge. The monomers contain a heavy subunit (55–60 kDa), which is covalently linked to the heme, and a light subunit (10.5–15 kDa). The properties of MPO differ significantly from those of other mammalian peroxidases like lactoperoxidase

(LPO), eosinophil peroxidase (EPO), and thyroid peroxidase (TPO). The visible absorption bands associated with the heme prosthetic group are red-shifted: the Soret band of MPO lies at 428 nm (Wever and Plat, 1981), whereas other mammalian peroxidases have Soret bands around 413 nm. Soret excitation Raman spectra of MPO are more complex, especially in the oxidation state marker region (1340–1380 cm^{-1}) (Sibbett and Hurst, 1984; Babcock et al., 1985; Stump et al., 1987). Furthermore, MPO is the only peroxidase that is able to catalyze the oxidation of chloride into hypochlorite at a significant rate. Crystal structure data (Zeng et al., 1992; Fenna et al., 1995) show that several residues are in close proximity to the heme group. Recently it has been reported that a mutation of Met²⁴³, one of these residues, has a large effect on the optical absorbance spectrum as well as the resonance Raman spectrum (Kooter et al., 1997). Kooter et al. concluded from these results that MPO has an iron porphyrin-like chromophore, and explained the red-shifted Soret band by the interaction of Met²⁴³ with the prosthetic group via a special sulfonium linkage and the presence of ester linkages between hydroxylated methyl groups on the heme and glutamate and aspartate residues, respectively.

Neutrophil cytochrome b_{558} consists of a FAD (flavin-adenine dinucleotide)-containing flavoprotein, a heme, and the NADPH-binding site (Segal et al., 1992; Rotrosen et al., 1992; Sumimoto et al., 1992). Raman spectra obtained upon Soret excitation (413 nm) indicated that the heme is a low-spin six-coordinate protoporphyrin IX (Hurst et al., 1991).

The importance of MPO in the host defense against invading organisms is not clear, because people with an MPO deficiency are in good health in most cases, although candidiasis, a fungal infection, has been reported to occur frequently in these people (Nauseef, 1990; Lehrer and

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Cline, 1969). Patients with chronic granulomatous disease (CGD), caused by a defect in, or the absence of, cytochrome b_{558} , suffer from severe, recurrent infections (Baehner and Nathan, 1967; Curnutte et al., 1974; Smith and Curnutte, 1991). This observation indicates that cytochrome b_{558} is necessary for a properly functioning immune system.

Confocal Raman microscopy might provide more insight into the exact role of both proteins in the neutrophil immune response. Raman spectra of a very small volume (0.5 fl) can be obtained with this technique. These spectra contain information about the vibrational states of the molecules in the measurement volume. The energy of these vibrational states depends on the mass of the atoms involved in the vibration and the strength of the bond between these atoms. Therefore, a Raman spectrum is specific for a particular molecule or molecular group and reveals the molecular composition in the measurement volume.

The technique is very suitable for investigating living cells under physiological conditions, because no specific treatment of the sample, like the introduction of extrinsic labels, is necessary. Raman microspectra have been published for tissue (Manoharan et al., 1996), chromosomes (Puppels et al., 1992), and living cells (Puppels et al., 1990, 1993) under physiological conditions. Spectra of the cytoplasmic region of neutrophils at an excitation wavelength of 660 nm showed a strong contribution of MPO (Puppels et al., 1991). Resonance Raman spectra of frozen pellets of neutrophilic granulocytes at 413.1-nm excitation mainly contained Raman bands originating from cytochrome b_{558} (Hurst et al., 1991).

In resonance Raman (Vickers et al., 1991), an excitation wavelength is used that lies in or close to an absorption band of the molecule of interest. The resonance Raman effect leads to a considerable enhancement of the Raman signal of this molecule. A strong enhancement of the cytochrome b_{558} signal and a less strong enhancement of the MPO signal is thus expected with an excitation wavelength of 413.1 nm, as its Soret bands are found at 413 nm (Isogai et al., 1991) and 430 nm (Wever and Plat, 1981), respectively.

The Raman spectra of neutrophilic granulocytes are rather complex. Therefore we have started by measuring isolated specific and azurophilic granule fractions. The spectra of these granule fractions appeared to correspond well to published Raman spectra of isolated cytochrome b_{558} (Hurst et al., 1991) and MPO (Sibbett and Hurst, 1984; Babcock et al., 1985; Stump et al., 1987), respectively. Raman spectra of MPO-deficient and cytochrome b_{558} -deficient neutrophils from a patient with CGD were measured at 413.1-nm excitation, to determine whether additional Raman bands originating from compounds other than MPO or cytochrome b_{558} were present in the spectra of whole cells. Furthermore, normal neutrophils were measured, and their resonance Raman spectra were compared with the spectra of the separated granule fractions.

Similar measurements were performed with specific and azurophilic granule fractions and MPO-deficient and cytochrome b_{558} -deficient neutrophils after reduction of the

redox centers with sodium dithionite. These measurements showed that clear spectral changes occur after reduction of the redox centers of MPO and/or cytochrome b_{558} .

MATERIALS AND METHODS

Peripheral blood neutrophils were isolated from fresh heparinized blood as described previously (Yazdanbakhsh et al., 1987). Neutrophils without MPO were obtained from a donor with a complete MPO deficiency, and granulocytes without cytochrome b_{558} were from a patient with CGD. Quartz plates were incubated overnight with 0.01% poly-L-lysine (PLL) (P-1274; Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) at 4°C. A few drops of 2×10^6 neutrophils/ml suspended in culture medium (RPMI 1640 + 25 mM HEPES without phenol red; Seromed) with 3% fetal calf serum (FCS) (011-06180; GIBCO, Paisley, Scotland) were put on a PLL-coated quartz glass. After an incubation of 10 min at 37°C, the cells stuck to the quartz, the neutrophil containing quartz glass was put into a petri dish (35 mm), and 2 ml RPMI + FCS was added. During the measurements the sample was kept at 37°C.

Specific and azurophilic granule fractions were obtained after sonication of neutrophils, followed by discontinuous sucrose gradient centrifugation (15/40/52/60% weight/volume sucrose) as described in detail by Bolscher et al. (1990). A few drops of granule suspension were put on a PLL-coated quartz glass, and after 10 min PBS was added. The Raman spectra of the granules were obtained by focusing the laser beam on a large group of granules. The spectra were corrected for the fact that the separation of the granules was not 100%, and ~20 spectra were averaged to improve the signal-to-noise ratio. The resulting spectra still contained some glucose, quartz, and sodium dithionite signal that was subtracted. A Zeiss 63× water immersion objective with a numerical aperture (NA) of 1.2 was used for the measurements on both neutrophils and granule fractions.

Human myeloperoxidase was isolated and purified from leukocytes as described by Bakkenist et al. (1987). A solution of MPO in a 100 mM potassium phosphate buffer was measured in 0.6×0.6 mm square capillaries, with a 0.12-mm wall thickness. A 40× objective (Spindler and Hoyer GmbH and Co., Göttingen, Germany) was used.

Raman spectra were measured with a confocal Raman microspectrometer described by Sijtsema et al. (1998). A schematic representation of the set-up is shown in Fig. 1. The 413.1-nm line of a krypton laser (Coherent, Innova 90-K) is reflected by a beamsplitter (reflection, 30%; transmission, 70% for 413 nm) and is focused on the sample by a microscope objective (Zeiss Plan Neofluar, 63×, NA 1.2, water immersion). The scattered light is collected by the same objective and transmitted through the beamsplitter. A holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI) is used to suppress reflected laser light and Rayleigh scattered light. The scattered

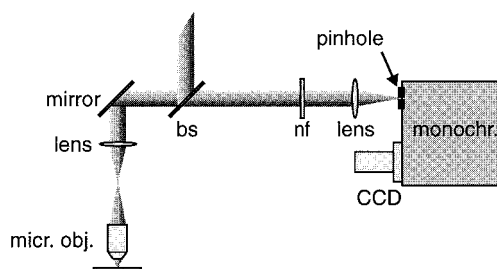


FIGURE 1 Schematic representation of the confocal Raman microspectrometer. A laser beam is reflected by a beam splitter (bs) and focused to a diffraction-limited spot by a microscope objective. The backscattered light is transmitted by the beam splitter and is focused onto a confocal pinhole at the entrance of a monochromator. Confocal Raman spectra are detected by means of a charge-coupled device (CCD) camera positioned at the multichannel exit port of the monochromator. A notch filter (nf) is used to suppress light with the laser frequency.

light is focused on a pinhole ($50\ \mu\text{m}$) positioned at the entrance of a Jobin-Yvon HR460 imaging spectrograph/monochromator (ISA; Jobin-Yvon, Paris, France) containing a blazed holographic grating with 1200 gr/mm (630-nm blaze). A Princeton liquid nitrogen-cooled CCD detector containing a back-illuminated chip with 1100×330 pixels of $24 \times 24\ \mu\text{m}^2$ (LN/CCD 1100 PB/VISAR; Princeton Instruments, Trenton, NJ) is placed in the focal plane of the spectrograph exit port and is used to measure Raman spectra of a small sample volume. The spatial resolution of the set-up was determined for two limiting cases: a thin layer and a small sphere. The resolution (full width at half-maximum) for a small sphere (diameter $0.282\ \mu\text{m}$) was $0.37\ \mu\text{m}$ in the lateral direction and $1.2\ \mu\text{m}$ in the axial direction. The axial resolution for a thin layer (thickness $0.28\ \mu\text{m}$) appeared to be $3.6\ \mu\text{m}$ (Sijtsema et al., 1998). The axial resolution for cellular samples will be somewhere between $1.2\ \mu\text{m}$ and $3.6\ \mu\text{m}$.

In the Raman measurements on neutrophils and granule fractions, it was observed that the MPO and cytochrome b_{558} signal diminished fast during the illumination of the sample. This bleaching is probably caused by photodestruction of the MPO and cytochrome b_{558} . We have diminished the effect of bleaching in our measurements by optimizing the measurement time and the laser power. We found that with a laser power of 0.5 mW on the sample and a measurement time of less than 15 s, no decrease in the Raman signal could be observed. Therefore, we assumed that the effects of photodestruction of the proteins are negligible if every cell is measured only once during 10 s with a laser power of 0.5 mW.

The neutrophil spectra were measured by focusing the laser beam in an area with a large concentration of granules inside the cell. The spectra of 10–15 measurements on different cells were averaged to improve the signal-to-noise ratio. Quartz and buffer signals were subtracted.

RESULTS

In Fig. 2 resonance Raman spectra with an excitation wavelength of 413.1 nm of azurophilic granules (Fig. 2 *a*) and

CGD neutrophils without cytochrome b_{558} (Fig. 2 *b*) have been compared with those of isolated MPO (Fig. 2 *c*). The three spectra show a large similarity. The Raman bands of isolated MPO are compiled in Table 1, column 1. The only differences are the band at $1663\ \text{cm}^{-1}$ and a shoulder at the right-hand side of the $1552\ \text{cm}^{-1}$ band in the granule, and the CGD spectra, which cannot be recognized in the isolated MPO spectrum.

In Fig. 2, *d–f*, the spectra of the azurophilic granules, CGD neutrophils, and isolated MPO after reduction of the redox centers by sodium dithionite are presented. The band-positions in spectra *d* and *e* are similar to those of isolated MPO with a reduced redox center (Table 1, column 2); however, the relative intensity of the 1359 and $676\ \text{cm}^{-1}$ bands is larger in Fig. 2 *e* than in Fig. 1, *d* and *f*. Furthermore, an extra band around $1558\ \text{cm}^{-1}$ is present in Fig. 2, *d* and *e*. The bands at 980 and $1052\ \text{cm}^{-1}$ (indicated with an * in Fig. 2 *f*) originate from sodium dithionite.

Fig. 3 shows the resonance Raman spectra of specific granules (Fig. 3 *a*) and MPO-deficient neutrophils (Fig. 3 *b*). Spectra of the same species after reduction of the redox centers with sodium dithionite are presented in graphs *c* and *d*. The strongest bands in Fig. 3, *a* and *b*, correspond well to published bands of isolated cytochrome b_{558} (Hurst et al., 1991), which are summarized in Table 1, column 5. Spectra *c* and *d* show a large resemblance to the resonant Raman spectrum of reduced cytochrome b_{558} published by Hurst et al. (Table 1, column 6). The bands at 980 and $1050\ \text{cm}^{-1}$ (indicated with an *) originate from sodium dithionite. The

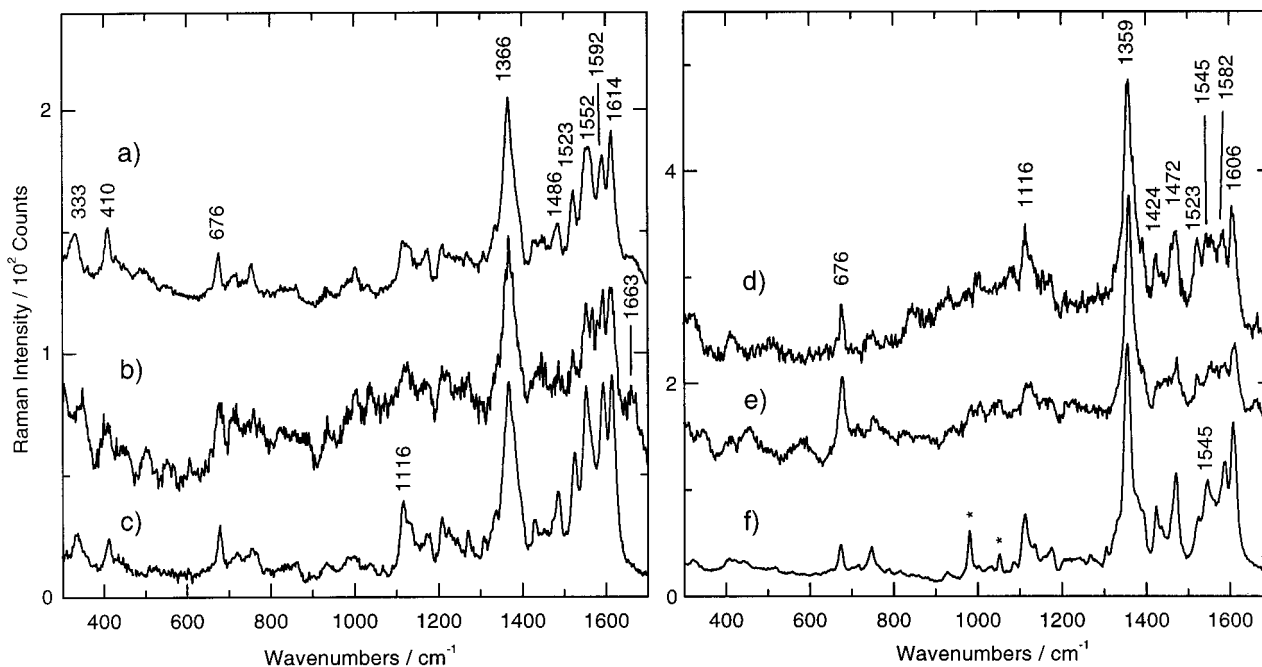


FIGURE 2 Resonance Raman spectra (413.1 nm excitation) of azurophilic granules (*a*), cytochrome b_{558} -deficient neutrophils (*b*), and a $15\ \mu\text{M}$ MPO solution in phosphate buffer pH 5 (*c*). Spectra *a–c* are scaled on the $1366\ \text{cm}^{-1}$ band and show mainly MPO-specific bands. Graphs *d–f* show spectra of the same species after reduction of the redox center and are scaled on the $1359\ \text{cm}^{-1}$ band. Spectra *d–f* are similar to spectra of reduced MPO. Bands indicated with an * in spectrum *f* originate from sodium dithionite.

TABLE 1 Comparison of strongest Raman bands of MPO, EPO, and cytochrome b_{558}

Oxidized MPO	Reduced MPO	Oxidized EPO	Assignment EPO	Oxidized Cyt. b_{558}	Reduced Cyt. b_{558}	Assignment Cyt. b_{558}
333	—	337	Vm*: $\delta(C_b C_\alpha C_\beta)^{\S}$	—	—	—
—	—	—	—	344	344	Vm: $\nu_8(C_b S)$
410	—	410	Vm: $\delta(C_b C_\alpha C_\beta)^{\ddagger}$	—	—	—
—	—	—	—	419	—	Vm: $\delta(C_b C_\alpha C_\beta)^{\S}$
676	676	675	Pm [#] : $\nu_7(\delta(C_b C_\alpha N))$	676	676	Pm: $\nu_7(\delta(C_b C_\alpha N))$
—	748	—	—	—	748	Pm: $\nu_{16}(\delta(C_\alpha N C_\alpha))$
1116	1116	1123	Pm: $\nu_{44}(C_b S)$	—	—	—
—	—	—	—	1131	1130	Pm: $\nu_6 + \nu_8$
1366	1359	1369	Pm: $\nu_4(C_\alpha N)$	1375	1359	Pm: $\nu_4(C_\alpha N)$
1376	—	—	—	—	—	—
—	1424	1425	Vm: $\delta_s(=CH_2)_{(1)}$	—	—	—
—	—	—	—	—	1472	Pm: $\nu_{28}(C_\alpha C_m)$
1486	1472	1485	Pm: $\nu_3(C_\alpha C_m)$	1507	—	Pm: $\nu_3(C_\alpha C_m)$
1523	1523	1520	Pm: $\nu_{38}(C_\alpha C_m)$	—	—	—
1552	1545	—	—	—	—	—
—	—	1561	Pm: $\nu_2(C_b C_b)$	—	—	—
1592	1582	1583	Pm: $\nu_{37}(C_b C_b)$	1582	1585	Pm: $\nu_2(C_b C_b)$
1614	1606	1616	Vm: $\nu(C=C)$	1622	1614	Vm: $\nu(C=C)$

Assignments of EPO and cytochrome b_{558} bands are taken from Salmaso et al. (1994) and Hurst et al. (1991), respectively.

*Vm: Vinyl modes.

[#]Pm: Porphyrin modes.

[§]In-phase vinyl mode.

[¶]Out-of-phase vinyl modes.

spectrum of MPO-deficient neutrophils with reduced redox centers (Fig. 3 *d*) contains a band at 1662 cm^{-1} that cannot be recognized in the spectrum of reduced specific granules (Fig. 3 *c*).

In Fig. 4 a linear combination of the Raman spectra of azurophilic and specific granules (Fig. 4 *a*) is compared with the resonance Raman spectrum of neutrophilic granulocytes with an excitation wavelength of 413.1 nm (Fig. 4

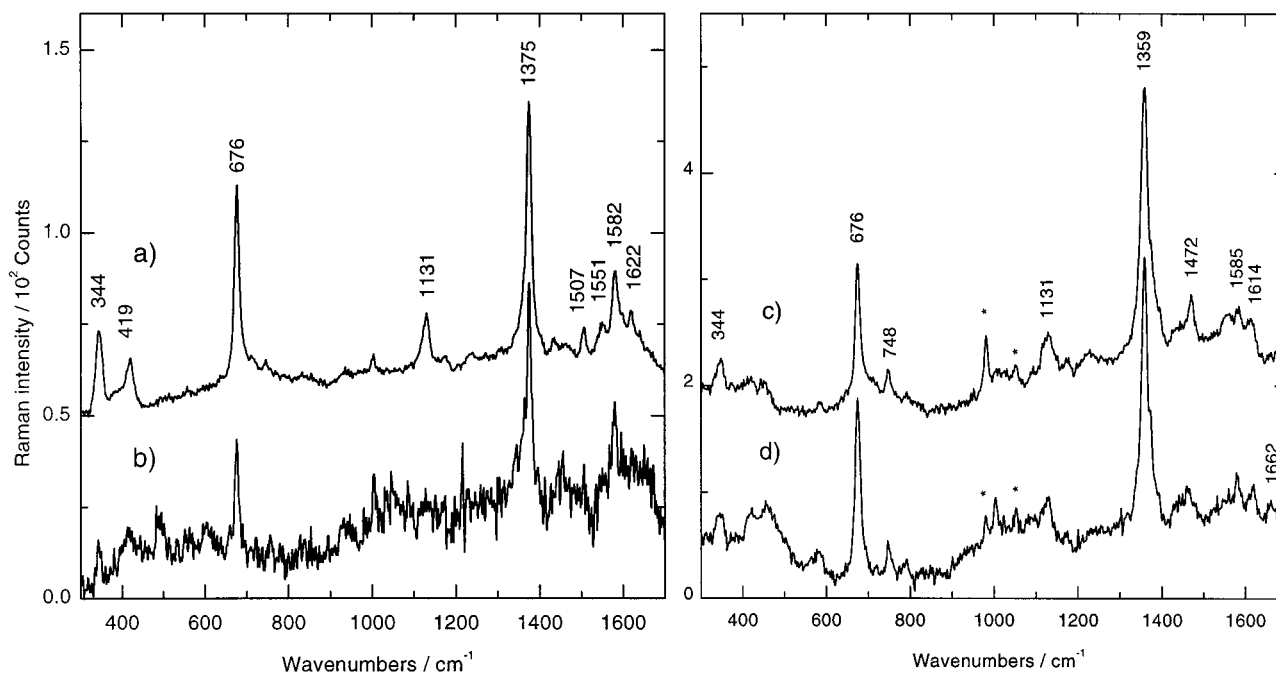


FIGURE 3 Resonance Raman spectra (413.1 nm excitation) of specific granules (*a*), neutrophils without MPO (*b*), and the same species after reduction of the redox centers (*c*, *d*). The spectra correspond well with published spectra of isolated cytochrome b_{558} (Hurst et al., 1991), which indicates that the Raman signal of specific granules and MPO-deficient neutrophils mainly originates from cytochrome b_{558} . Spectra *a* and *b* are scaled on the 1375 cm^{-1} band, and spectra *c* and *d* on the 1359 cm^{-1} band. Bands in *c* and *d* indicated with an * originate from sodium dithionite.

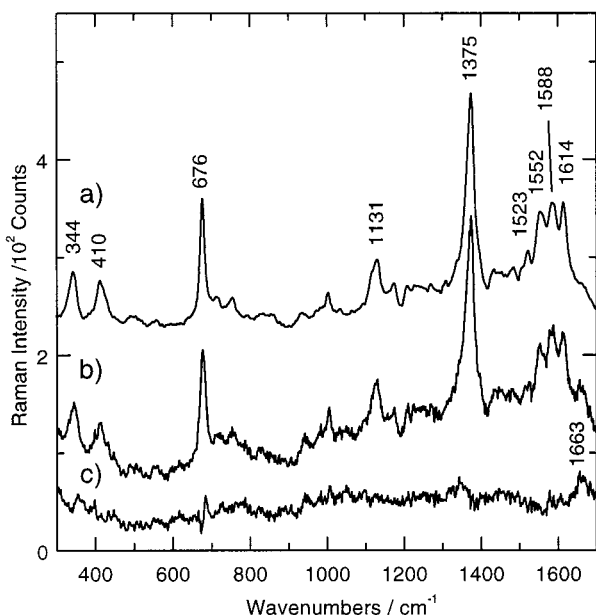


FIGURE 4 Comparison of the sum of the azurophilic granules spectrum and the specific granules spectrum (*a*) with a normal neutrophil spectrum (*b*, average of 13 measurements) with an excitation wavelength of 413.1 nm. The spectra are scaled on the 1375 cm^{-1} band; spectrum *c* is the difference between *a* and *b*. The figure shows that the Raman spectrum of normal neutrophils mainly contains signals originating from MPO and cytochrome b_{558} with this excitation wavelength.

b). In Fig. 4 *c* the difference between Fig. 4 *a* and Fig. 4 *b* is shown. It is obvious that the spectrum of neutrophils corresponds very well to the combination of the spectra of the specific and azurophilic granules.

DISCUSSION

An assignment of the Raman bands of cytochrome b_{558} was published by Hurst et al. (1991). No complete assignment of the Raman bands of MPO has been published, as far as we know. The origin of the differences between the resonant Raman spectra of MPO and those of other mammalian peroxidases like LPO and EPO is not fully understood yet. The Raman spectrum of MPO is more complicated, with multiple bands in the ν_4 region (oxidation state marker; 1350–1380 cm^{-1}). Several authors have suggested that these differences can be explained by the symmetry-lowering effects of a chlorin-like structure of the prosthetic group (Sibbett and Hurst, 1984; Babcock et al., 1985; Andersson et al., 1984). However, in recent publications it was shown that mutations of residues in close proximity to the prosthetic group of MPO affect the resonant Raman spectrum significantly. After a mutation of the Met²⁴³ residue to Gln, the resonant Raman spectrum of MPO is rather similar to the spectra of LPO and EPO (Kooter et al., 1997). These results indicate that MPO contains an iron-protoporphyrin-like heme, and that the spectral differences can be explained by an interaction of the protein matrix with the chromophore. In Table 1 a comparison is given of resonant

Raman bands of MPO, EPO, and cytochrome b_{558} . The assignments of resonant Raman bands of EPO was taken from Salmaso et al. (1994), and the assignments of cytochrome b_{558} bands from Hurst et al. (1991).

Fig. 2 shows the large similarity of the resonance Raman spectra of azurophilic granules, CGD neutrophils, and isolated MPO. One difference that can be recognized is the band around 1663 cm^{-1} in Fig. 2, *b* and *e*, that can be attributed to an amide I vibration of protein in the cell. Furthermore, in Fig. 2, *a* and *b*, the band at 1552 is broader or comprises a stronger shoulder than in Fig. 2 *c*. Moreover, in the spectra of the azurophilic granules and CGD neutrophils after reduction of the redox centers (Fig. 2, *d* and *e*), an extra band around 1558 cm^{-1} can be recognized. This extra band is most probably caused by Raman scattering originating from oxygen molecules, which is known to have a Raman band around 1558 cm^{-1} . This scattering by oxygen molecules occurs along the pathway of the laser beam through air. A small part of this scattered light can reach the detector. Because the signal levels in the spectra of isolated MPO are much higher, this small oxygen signal cannot be recognized there.

If the graphs 1 *a–c* are compared with 1 *d–f*, it is clear that a reduction of the redox centers caused distinct changes in the Raman spectrum of the azurophilic granules and CGD neutrophils. A reduction of the redox centers results in a shift of the 1366, 1486, 1552, 1592, and 1614 cm^{-1} bands to 1359, 1472, 1545, 1582, and 1606 cm^{-1} , respectively.

The spectra of the oxidized and reduced specific granules in Fig. 3 correspond very well with published spectra of isolated cytochrome b_{558} (Hurst et al., 1991). Furthermore, the spectra of the neutrophils without MPO are similar to the spectra of the specific granules, apart from the protein band at 1662 cm^{-1} in Fig. 3 *d*. Therefore, we conclude from the spectra in Figs. 1 and 2 that the neutrophils do not contain components other than MPO and cytochrome b_{558} that contribute substantially to the Raman spectrum measured with 413.1-nm excitation.

The reduction of the redox center in cytochrome b_{558} can be recognized clearly in the Raman spectra of specific granules and neutrophils without MPO. After reduction, the 1375, 1507, and 1622 cm^{-1} bands shift to 1359, 1472, and 1614 cm^{-1} , respectively.

A comparison of the spectra of azurophilic granules and specific granules excited at 413.1 nm shows that the granules can be distinguished based on their Raman spectrum. The most striking differences between Figs. 2 *a* and 3 *a* are the bands at 333, 410, 1366, 1486, 1523, 1592, and 1614 cm^{-1} in Fig. 2 *a*, whereas Fig. 3 *a* contains bands at 344, 419, 1131, 1375, and 1582 cm^{-1} . Furthermore, the relative intensity of the 676 cm^{-1} band is much larger in the specific granules than in the azurophilic granules. The difference in the spectra after reduction of the redox centers is less obvious. In Fig. 2 *d* the relative intensity of the 676 cm^{-1} band is weaker than in Fig. 3 *c*, and bands can be recognized at 1424, 1523, and 1606 cm^{-1} that are not present in Fig. 3

c, whereas the 1614 cm^{-1} band of Fig. 3 *c* is not present in Fig. 2 *d*.

In Fig. 4 it is shown that the Raman spectrum of normal neutrophils can be fitted with the spectra of specific and azurophilic granules. Therefore, this figure shows that in normal cells the Raman spectrum measured with a 413.1-nm excitation contains mainly signal originating from MPO and cytochrome b_{558} .

Our results show that the contribution of reduced and oxidized MPO as well as reduced and oxidized cytochrome b_{558} can be distinguished in the Raman spectra of living neutrophils. This is a very interesting result, because this means that processes inside living neutrophils, leading to a partial reduction of MPO or cytochrome b_{558} , can be followed with Raman microspectroscopy. It is known that a reduction of cytochrome b_{558} occurs after activation of neutrophils with opsonized particles or soluble activators (Borregaard, 1988; Cross and Curnutte, 1995). Furthermore, a reduction of MPO in activated neutrophils has been reported (Winterbourn et al., 1985). Therefore, Raman microspectroscopy seems a very suitable technique for investigating reactions inside activated neutrophils. More knowledge about reactions occurring inside activated neutrophils may lead to more insight into the exact role of MPO and cytochrome b_{558} in the human immune response.

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