The association of FAD with the cytochrome b_{-245} of human neutrophils

Andrew R. CROSS and Owen T. G. JONES

Department of Biochemistry, The Medical School, University of Bristol, Bristol BS8 1TD, U.K.

and Rudolfo GARCIA and Anthony W. SEGAL

Department of Haematology, School of Medicine, University College, London W.C.1., U.K.

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A plasma membrane fraction prepared from human neutrophils had a fluorescence resembling that of a fluorescent flavoprotein, with emission maximum near 520 nm and excitation maxima near 380 and 460 nm. The fluorescence emission and excitation properties of Triton N-101-solubilized membrane fraction resembled those of FAD. FAD was present in the membranes at a concentration of 417 pmol/mg of protein and cytochrome b_{-245} at a concentration of 407 pmol/mg of protein. In a 110-fold purified preparation of cytochrome b_{-245} the ratio of FAD:cytochrome b was 1:1. Analytical gradient centrifugation of neutrophil homogenates shows a coincidence of two cytochrome b peaks and two peaks of fluorescence, corresponding with plasma membrane and specific granule fractions; most of the FAD was non-fluorescent and located in fractions lighter than the plasma membrane. Plasma membrane fractions prepared from neutrophils of patients suffering from the X-linked form of chronic granulomatous disease lacked cytochrome b and contained 194 pmol of FAD/mg of protein; plasma membrane fractions prepared from neutrophils of patients with the autosomal recessive form of chronic granulomatous disease contained both cytochrome b_{-245} and FAD in the normal range of concentrations in a ratio of 1:1. Phagocytic vesicles were prepared from normal neutrophils and found to contain FAD and cytochrome b in a ratio 2.22:1, suggesting that activation of neutrophils may involve the incorporation of an additional flavin into the membrane. Under anaerobic conditions in the presence of EDTA to act as an electron donor to a flavin, the cytochrome b_{-245} of neutrophil membranes was partly (12%) photoreducible, an effect increased to 100% by the addition of FMN. The extent of reduction of cytochrome b in an anaerobic neutrophil homogenate containing NADH increased from 30% to 70% on illumination. We suggest that these results indicate a close association between FAD and cytochrome b_{-245} and support a scheme for electron transport thus:

Neutrophil polymorphonuclear leucocytes (neutrophils) are phagocytic cells which form part of the mammalian host defence system against bacterial infection. When neutrophils phagocytose microbes or are exposed to phorbol myristate acetate they greatly increase their oxygen consumption and produced superoxide ($\text{O}_2^{\cdot-}$), peroxide (O_2^{2-}) and hydroxyl radical ($\text{OH}^{\cdot}$) in 'respiration' which does not appear directly to involve mitochondrial electron transport (Sbarra & Karnovsky, 1959). Much of the early literature on this topic has been reviewed by

Babior (1978) and Badwey & Karnovsky (1980). The increased respiration of phagocytosis (Baldridge & Gerard, 1933) coincides with increased glucose oxidation via the pentose phosphate pathway (Sbarra & Karnovsky, 1959), and membranes prepared from stimulated neutrophils catalyse the reduction of oxygen to $\text{O}_2^{\cdot-}$ using NADPH as electron donor (Iyer & Quastel, 1963; Zatti & Rossi, 1956).

The $\text{O}_2^{\cdot-}$ -forming activity of zymosan-stimulated human neutrophils was lost in the presence of

detergent but could be stabilized by the addition of FAD (Babior & Kipnes, 1977), and this has led to the description of the NADPH-dependent $O_2^{\cdot-}$ -generating system as a flavoprotein (Gabig & Babior, 1979). Similar conclusions were drawn by Light *et al.* (1981), who found a similar requirement for FAD in detergent-solubilized $O_2^{\cdot-}$ -forming systems from human neutrophils and observed their sensitivity to inhibition by FAD analogues.

We have noted that the plasma membranes of human neutrophils are associated with a low potential cytochrome *b* (Segal & Jones, 1978; Cross *et al.*, 1981), which is absent from the neutrophils of some patients suffering from the X-linked form of chronic granulomatous disease, where there is a lowered resistance to microbial infection and where the superoxide-generating system is absent. In another group of patients (with autosomal recessive inheritance) the cytochrome *b*₋₂₄₅ is present but is not reduced upon stimulating an anaerobic suspension of the cells, a reaction characteristic of normal cells (Segal & Jones, 1979b). We have found cytochrome *b*₋₂₄₅ to be present in a number of different human phagocytic cells, e.g. macrophages, monocytes and eosinophils (Segal *et al.*, 1981) and in neutrophil membranes prepared from ox, horse and pig blood (Cross *et al.*, 1982). In the present communication we present evidence that FAD is associated with cytochrome *b*₋₂₄₅ in the plasma membrane fraction of human neutrophils.

Materials and methods

Preparation of human neutrophils and neutrophil plasma membranes

These methods have been fully described in Segal & Jones (1979a).

Analytical gradient fractionation and assay of marker enzymes

Methods were as described in Segal & Jones (1979a).

Preparation of neutrophils from patients with chronic granulomatous disease

Details of the diagnosis of the X-linked and autosomal recessive variants of chronic granulomatous disease are described elsewhere (Segal *et al.*, 1983).

Fluorimetry

Excitation and emission spectra were measured by using a Farrand Spectrofluorimeter, mark 1.

Cytochrome *b*₋₂₄₅

This cytochrome was assayed by reduced-minus-oxidized difference spectroscopy using $\Delta\epsilon_{559-540} = 21.6 \text{ cm}^{-1} \cdot \text{mm}^{-1}$ (Cross *et al.*, 1982).

Assay of FAD and FMN

The method of Faeder & Seigel (1976) was used. Flavins were extracted by heating for 5 min in a water bath at 100°C before assay of fluorescence.

Results

Detection of flavin in neutrophil plasma membranes

Examination of the fluorescence of the plasma membrane fraction of human neutrophils revealed an emission band at around 520 nm with excitation maxima near 380 and 460 nm. Although light-scattering effects prevented precise determination of the excitation and emission spectra these were similar to those described for fluorescent flavoproteins (see for example, Ghisla *et al.*, 1974).

The similarities of the fluorescence excitation spectra of solubilized membranes to the fluorescence spectrum of FAD were striking; there were major bands near 380 nm and 460 nm in each (Fig. 1). Analysis of the flavin content of the purified membranes showed that flavin was present and that over 90% of the flavin was FAD. It is likely that the small amount of FMN present arises from breakdown of FAD during extraction and assay. The amount of FAD in neutrophil membranes was apparently unaltered in patients with the autosomal

Fig. 1. Fluorescence excitation spectrum of a solubilized cytochrome *b*₋₂₄₅ preparation

Human plasma membranes were extracted with a buffer containing KCl (100 mM), dithiothreitol (60 μ M), Tris acetate (60 μ M), EDTA (0.6 mM), Mops (4-morpholinepropane sulphonic acid) (50 mM), glycerol (12%, v/v) and Triton N-101 (0.3%, v/v), pH 7.0. Insoluble material was removed by centrifugation; the soluble fraction contained 1 mol of cytochrome *b*₋₂₄₅ per mol of FAD, and 80% of the cytochrome *b*₋₂₄₅ was extracted from the membrane. Excitation spectrum was measured by monitoring fluorescence at 520 nm and is compared with that of FAD dissolved in the same buffer.

Table 1. FAD content of plasma membrane fraction from normal and chronic granulomatous disease (CGD) human neutrophils

Details of membrane preparation and assay for FAD are given in the Materials and methods section. The cytochrome b_{-245} content of normal membranes was 407 nmol · mg of protein⁻¹.

Source of membranes	FAD content (pmol · mg of protein ⁻¹)
Normal, human	417 ± 38 (n = 7)
Autosomal recessive CGD patient	411 ± 58 (n = 3)
X-linked CGD patient	194 ± 30 (n = 3)

recessive form of chronic granulomatous disease, where we have found that cytochrome b_{-245} is present (Segal & Jones, 1979b), but was decreased from about 400 to about 200 pmol of FAD · mg of protein⁻¹ in membranes from patients with the X-linked variety of chronic granulomatous disease (Table 1). These latter patients lack cytochrome b_{-245} (Segal & Jones, 1979b, 1980) and thus it appears that there may be a close association between FAD and cytochrome b_{-245} . It was also found that the FAD-like fluorescence in intact membranes of neutrophils from X-linked chronic granulomatous disease patients was only 40% of normal. This close relationship between cytochrome b_{-245} and FAD is supported by the results of an analytical gradient fractionation of a homogenate of normal human neutrophils (Fig. 2). There are two peaks of cytochrome b , corresponding to the plasma membrane and the specific granules (Segal & Jones, 1979a). No cytochrome oxidase (an indicator of mitochondrial contamination) was detected in spectra from either peak of cytochrome b . The most intense flavin fluorescence coincides with the cytochrome b_{-245} distribution. In the fractions richest in cytochrome b_{-245} the ratio of flavin to cytochrome b_{-245} approaches closely 1:1. Analysis of 110-fold purified cytochrome b_{-245} , prepared by extraction of neutrophil membranes in 1% Triton N-101, followed by chromatography on a column of heparin-Agarose and made available to us by Dr. Angela Harper (Department of Haematology, University College Hospital) gave an FAD:cytochrome b_{-245} ratio of 1:1.

Flavin content of phagocytic vacuole membranes

After neutrophils were stimulated by latex particles coated with immunoglobulin G the resulting vacuoles were collected by density gradient centrifugation of the cell homogenates (Segal & Jones, 1980). The vacuoles, formed from invaginations of the plasma membrane, contained flavin and cytochrome b_{-245} in a ratio $2.22 \pm 0.15:1$ (n = 5), higher than that found in plasma membranes.

Fig. 2. Analytical subcellular fractionation of human neutrophils on sucrose gradients

The activities of the fractions were assayed as described in the Materials and methods section and in Segal & Jones (1979a).

Reduction of cytochrome b_{-245} by photoreduced flavin

It is known that flavoproteins can be reduced by light under anaerobic conditions, using EDTA as electron donor (Massey & Palmer, 1966). In the case of the flavocytochrome b from *Neurospora crassa* or *Avena* coleoptiles, the flavocytochrome acts as a photoreceptor and direct photoreduction of the cytochrome has been described (Munoz & Butler, 1975; Brain *et al.*, 1977; Goldsmith *et al.*, 1980) using light absorbed by the flavin. We have attempted to achieve photoreduction of the cytochrome b_{-245} of neutrophil membranes to see if there is a close relationship between flavin and haem. Illumination of a suspension of membranes in 5 mM-EDTA with a 1000 W lamp at a distance of 15 cm induced a slight (approx. 12%) reduction of the cytochrome when glucose oxidase and glucose were added as oxygen scavengers to the anaerobic

cuvette which had been repeatedly evacuated and gassed with oxygen-free nitrogen. However, when FMN ($5\mu\text{M}$) was added to the cuvette complete reduction of the cytochrome b_{-245} was achieved within 2–3 min. FAD was much less effective in promoting photoreduction, in agreement with the observations on the ease of photoreduction of FMN and FAD and of FAD-containing flavoproteins (MacCormick, 1968; MacCormick *et al.*, 1967).

When a homogenate of neutrophils was incubated anaerobically with NADH, cytochrome b_{-245} became about 30% reduced. Illumination of this sample caused further reduction, to about 70%; transfer to dark caused a slow decline in the extent of reduction to about 48% (Fig. 3).

Discussion

Neutrophil membranes contain an electron transport system involved in the production of oxygen radicals from reduced substrates. Our view of the organization of this system in the plasma membrane is given in Scheme 1 and we review below the evidence in its support.

Fig. 3. Effect of illumination on the extent of reduction of cytochrome b of human neutrophils

A total homogenate of neutrophils in 50 mM-Mops/50 mM-KCl, pH 7.0, was incubated in an anaerobic cuvette containing glucose oxidase (100 units) and glucose (10 mg/ml) as oxygen scavengers and NADH (2 mM) and EDTA (0.6 mM). Where indicated by arrows a 1 kW lamp illuminated the suspension with white light. Extent of reduction of cytochrome b was measured at 559 nm–540 nm in a split-beam spectrophotometer.

Cytochrome b_{-245} becomes reduced when human neutrophils are stimulated by phorbol myristate acetate under anaerobic conditions (Segal & Jones, 1979b) and is rapidly oxidized upon reintroduction of air. Cytochrome b_{-245} of isolated membranes is rapidly oxidized by air ($t_{1/2}$ less than 5 ms; Cross *et al.*, 1982) and, as is common with oxidases, forms a photodissociable CO complex (Cross *et al.*, 1981, 1982). The oxidation–reduction midpoint potential of cytochrome b_{-245} is sufficiently low for it to reduce O_2 to $\text{O}_2^{\cdot-}$ at a fast rate.

The nature of the reductant for superoxide generation has been the subject of controversy. Cagan & Karnovsky (1964) proposed NADH as electron donor whereas Patriarca *et al.* (1971) and, more recently, Babior (see review by Babior, 1978) have favoured NADPH. We have found that both NADH and NADPH caused the reduction of cytochrome b_{-245} in isolated membranes under anaerobic conditions (Cross *et al.*, 1982) but have not investigated the relative affinity of the system for these two donors.

It is likely that a flavin will be involved in a pathway between NAD(P)H and a cytochrome b , a pathway linking two-electron donors to a one-electron acceptor. An interesting example is the NADH:cytochrome b_5 reductase of microsomes, an FAD-containing enzyme (Strittmatter & Velick, 1957). The superoxide-generating system of neutrophils has been described as a flavoprotein because the activity of detergent-solubilized preparations is increased by added FAD (Gabig & Babior, 1979) and Cagan & Karnovsky (1964) very briefly mentioned that the neutrophil NADH oxidase might contain FAD. Some support for this was supplied by Light *et al.* (1981) who showed that 5-carba-5-deaza-FAD inhibited superoxide production by their solubilized, partially purified superoxide-generating systems and that this extract contained both cytochrome b and FAD. Our results implicate FAD as a component in the specialized electron transport system of the neutrophil plasma membrane from NADH(P)H to oxygen. We suggest that the system has the form shown in Scheme 1, where it is possible that FAD is normally attached to the cytochrome. Previously, little analysis of the flavin content of neutrophil homogenates or fractions has

Scheme 1. Representation of the plasma membrane electron transport system of human neutrophils
It is possible that the cytochrome b_{-245} is also the FAD-carrying protein.

been undertaken. Neutrophils contain few mitochondria and little endoplasmic reticulum and the cytochrome b_{-245} -linked system appears to be the major electron transport chain of the cell. Thus membrane-bound flavin found in abundance is likely to be associated with this system. We have now found that cytochrome b_{-245} is partly reduced following illumination of the membrane anaerobically, in the presence of EDTA to promote flavin reduction. The subcellular distribution of a fluorescent flavoprotein is similar to that of cytochrome b_{-245} . The molar ratio of flavin to cytochrome b_{-245} in purified membranes and in purified cytochrome b preparations is near 1:1. In phagocytic vacuoles (where some fusion of specific granules with plasma membrane will have taken place) the ratio is 2.3:1, and it is possible that this is part of the process of activating the respiratory burst.

Chronic granulomatous disease is a syndrome found in a heterogeneous group of patients with various defects, all of which result in the common malfunction of the oxidase system. It is possible that some of these patients will have an abnormality of the flavin, but we have been unable to demonstrate this in the seven autosomal recessive subjects that we have studied. However in all three of the male subjects with X-linked chronic granulomatous disease that we examined we found the membranes contained almost exactly half of the normal concentration of FAD (Table 1). This raises the possibility that two flavoproteins may be involved in the electron transport chain, and that in these patients who lack evidence of the cytochrome b_{-245} one of the flavins, possibly a component of a flavocytochrome, is missing.

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