Subcellular Localization of NAD(P)H Oxidase(s) in Human Neutrophilic Polymorphonuclear Leucocytes

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NADH and NADPH oxidase activities in a homogenate of human neutrophils co-sediment in a linear sucrose density gradient under either velocity or isopycnic conditions of centrifugation. The position of these activities in the gradient does not correspond to any known subcellular granule or to the cell-membrane fraction. These data suggest that the oxidase activities may reside in a unique granule that has previously not been recognized.

During the process of phagocytosis by human neutrophils the cells undergo a remarkable change in metabolism. These metabolic alterations, collectively referred to as the 'respiratory burst', include increased oxygen consumption, increased oxidation of glucose via the hexose monophosphate shunt pathway, and the generation of active forms of oxygen, including hydrogen peroxide and superoxide anion (Cheson *et al.*, 1977).

Although most workers agree that the respiratory burst of human polymorphonuclear leucocytes is mediated by a reduced nicotinamide nucleotide oxidase (Cheson *et al.*, 1977), there remains considerable controversy over the nature of the enzyme. Although some workers consider the physiological substrate to be NADH (Briggs *et al.*, 1975; Segal & Peters, 1977), recent work is beginning to favour NADPH as the natural substrate (Cheson *et al.*, 1977; Curnutte *et al.*, 1975; Hohn & Lehrer, 1975; Iverson *et al.*, 1977). It is possible that the initiating oxidase is not absolutely specific and can utilize either reduced nicotinamide nucleotide as substrate. Still more confusion exists in the matter of subcellular localization of the enzyme(s). It is probably correct to state that most workers consider that the enzyme is located in the exterior of the plasma membrane (Briggs *et al.*, 1975; Segal & Peters, 1977; Takanaka & O'Brien, 1975; Goldstein *et al.*, 1975), but Patriarca *et al.* (1973) have published data suggesting that the NADPH oxidase of rabbit leucocytes is associated with the azurophil granule.

We recently presented some evidence from sucrose-gradient centrifugation that suggested that the human NADH and NADPH oxidase activities

were both found in a dense granule of the cell (Iverson *et al.*, 1977). These preliminary data were not complete for the following reasons. (1) Only one set of conditions (isopycnic centrifugation) was used for the separation; (2) only cells challenged with opsonized zymosan were used, and it is possible that activity was actually being determined in phagocytic vacuoles rather than granules; and (3) no reliable marker for the plasma membrane of the cell was then available. The present work extends the early observations by overcoming these limitations and supports the theory that the NAD(P)H oxidase of human leucocytes is granule-associated.

Methods

The procedure for cell isolation was previously described in detail (Iverson *et al.*, 1977). Briefly the erythrocytes were separated from heparinized venous blood by sedimentation with Plasmagel (HTI Corp., Buffalo, NY, U.S.A.), and the polymorphonuclear leucocytes separated from mononuclear cells with Ficoll/Hypaque. The few contaminating erythrocytes were then removed by hypo-osmotic lysis and the cells washed once with 0.34M-sucrose. Wright staining of slides prepared with a cytocentrifuge gave the following proportions of cell types: 93–96% neutrophils; 3–7% eosinophils; and 0–2% mononuclear cells.

When resting cells were used in the experiments the cells were concentrated to 3×10^8 /ml in 0.34M-sucrose and gently homogenized in a tight-fitting Potter-Elvehjem homogenizer with a Teflon pestle operated at 2400 rev./min. In some experiments, cells were allowed to phagocytose opsonized zymosan as previously described (Iverson *et al.*, 1977), before concentration to 3×10^8 /ml and subsequent homo-

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; Mg^{2+} -ATPase, Mg^{2+} -dependent adenosine triphosphatase.

genization. No attempt was made to achieve complete disruption of the cells, since this invariably led to clumping of the components and poor separation on the sucrose gradient; the usual extent of breakage, estimated by phase-contrast microscopy, was 60–80%. The whole homogenates (either resting or phagocytosing) were diluted to approx. 20ml with 0.34M-sucrose and centrifuged in the cold for 10min at 500g to remove intact cells, cell debris, nuclei, and zymosan particles. The resulting 500g-supernatant solutions were made 25% (w/w) in sucrose and diluted to 30ml with 25% sucrose for loading on the gradient.

A Beckman Ti-15 zonal rotor containing a linear 500ml gradient of 30–56% (w/w) sucrose with a 60% sucrose cushion was used in these experiments. The sample was applied to the gradient and overlaid with approx. 80ml of 25% sucrose. Under these conditions, the $r_{\min.}$ of this gradient was 2.70cm and the $r_{\max.}$ was 5.55cm. The gradients were centrifuged initially at 3000 rev./min. for 15min and then at 21000 rev./min for either 15min ($\int_0^t \omega^2 dt = 8.12 \times 10^9 s^{-1}$) or for 2h ($\int_0^t \omega^2 dt = 3.86 \times 10^{10} s^{-1}$) at 21°C in a Beckman model L2-65 ultracentrifuge. Fractions (10ml each) were collected from the top of the gradient by pumping 60% sucrose into the bottom. All gradient fractions were diluted with 0.16M-NaCl to a final sucrose concentration of approx. 11%, and the particulate material sedimented by centrifugation at 27000g for 60min. The resulting pellets were resuspended in 0.9% NaCl buffered with 5mM-Mes, pH7.4, for assay. Each fraction was assayed for lactoferrin, myeloperoxidase, nonspecific peroxidase, protein, and Mg^{2+} -ATPase as previously described (Harlan *et al.*, 1977). One gradient was also assayed for lysozyme as previously described (Iverson *et al.*, 1977). Oxidase activities were determined with either NADH or NADPH (Boehringer-Mannheim Corp., Munich, Germany) as substrate; the procedure was the same as previously reported (Iverson *et al.*, 1977), except that the buffer was changed from phosphate to 0.1M-Mes, pH6.0, to improve the buffer capacity at this pH. It is important to utilize the highest grade substrates available in order to ensure linearity of the reaction. Recoveries of all activities from the gradient were in the range of 60–80%, except those for the oxidases, which generally exceeded 100%, indicating some type of activation of the enzyme.

Results

Fig. 1 shows the results of a gradient obtained by using the 500g supernatant from cells that had phagocytosed opsonized zymosan. This gradient was run for 2h at 21000 rev./min and thus was similar to that we described previously (Iverson *et al.*, 1977), except that a zonal rotor was used in the present case. The present results are quite similar to those found

Fig. 1. Isopycnic centrifugation of leucocyte granules from phagocytosing cells

The gradients were prepared and assayed as described in the Methods section. In this particular experiment, NADPH oxidase was assayed at 0.20mM-NADPH, whereas NADH oxidation was measured at 0.80mM-NADH, for incubation times of 60min.

previously. Lactoferrin, a marker for the specific granule of the cell, was found primarily at a density of 1.19g/cm³, although there was a shoulder of activity deeper in the gradient. Myeloperoxidase, determined immunologically, and peroxidase, determined spectrophotometrically, co-sedimented to a density of 1.22g/cm³, marking the position of the azurophil granule. Lysozyme showed a broad band of distribution, consistent with its reported presence in both the specific and azurophil granules (Spitznagel *et al.*, 1974). The distribution of Mg^{2+} -ATPase, a marker enzyme for the plasma membrane, was quite broad, but the activity was generally higher in the gradient than that of myeloperoxidase. Both oxidase activities co-sedimented in the gradient. Although the patterns appeared to be skewed slightly to the left, the modal density was 1.24g/cm³, definitely greater than that of the azurophil granule.

We next attempted a velocity centrifugation (15 min at 21000 rev./min) to approach the question of size rather than density of the particles. A representative gradient of this type is shown in Fig. 2. As for Fig. 1, the gradient was run with the 500g supernatant from cells challenged with opsonized zymosan. Equilibrium conditions were not reached in this experiment, as indicated by the fact that both lactoferrin and myeloperoxidase were higher in the gradient than in

Fig. 2. Velocity centrifugation of leucocyte granules from phagocytosing cells

The gradients were prepared and assayed as described in the Methods section. NADPH and NADH oxidation were measured at 0.20mM-substrate for a 60-min incubation time.

Fig. 3. Isopycnic centrifugation of granules from resting leucocytes

Gradients were prepared and assayed as described in the Methods section. NAD(P)H oxidation was measured at 0.80mM-substrate for a 60-min incubation period.

Fig. 1, with modal densities of 1.16 and 1.19 g/cm³ respectively. Mg²⁺-ATPase was spread throughout the gradient. In contrast, both oxidase activities were found predominantly at a density of 1.24 g/cm³, as in the previous isopycnic gradient. Under these conditions, the oxidase activity appeared to separate into

Fig. 4. Velocity centrifugation of granules from resting leucocytes

Gradients were prepared and assayed as described in the Methods section. NAD(P)H oxidation was measured at 0.80mM-substrate for a 60-min incubation period.

a major and minor band, in contrast with the one lopsided band seen under isopycnic conditions. Since myeloperoxidase is known to oxidize nicotinamide nucleotides under these assay conditions, this may indicate that the oxidase activity of myeloperoxidase is being separated from an NAD(P)H oxidase associated with a very large, dense particle.

To rule out the possibility that the oxidase activities measured were associated with phagocytic vacuoles containing zymosan, the previous experiments were repeated with resting cells. Because of the generally lower oxidase activities seen in resting cells (Iverson *et al.*, 1977), these gradients were assayed at a reduced nicotinamide nucleotide concentration of 0.80mM rather than of 0.20mM. Fig. 3 shows the results of such an experiment run under isopycnic conditions. There was a clear separation of the specific and azurophil granules as indicated by the modal positions of lactoferrin and myeloperoxidase. The density of these peak components was precisely the same as that observed when using phagocytosing cells, as shown in Fig. 1. Again, the Mg²⁺-ATPase was widely distributed throughout the gradient. As in Fig. 1, the oxidase activities co-sedimented to a density of 1.24 g/cm³, deeper in the gradient than the peak of myeloperoxidase. Again, the curve was slightly skewed to the left.

Finally, the results of a velocity gradient using

resting cells are depicted in Fig. 4. The lactoferrin and myeloperoxidase are not as deep in the gradient as they are in Fig. 3, whereas the Mg^{2+} -ATPase is widely distributed. The main peak of oxidase activity is seen at a density of 1.24 g/cm^3 , with a suggestion of a minor peak, which appears to co-sediment with myeloperoxidase.

Discussion

The present results strongly support a granule location for the reduced nicotinamide nucleotide oxidase(s) of human neutrophilic polymorphonuclear leucocytes. Under conditions of either isopycnic or velocity sucrose-density centrifugation, oxidase activity toward both NADH and NADPH was found at a modal density of 1.24 g/cm^3 , deep in the gradient. The results under isopycnic conditions suggest the granule is quite dense, whereas the experiments using velocity conditions imply a relatively large granule. The position of the oxidase(s) in the gradient is distinct from that of the specific granule (determined by lactoferrin) or the azurophil granule (determined by myeloperoxidase). This separation is especially apparent in the velocity runs, where the other granules have not reached their equilibrium densities.

The distribution of the membrane marker enzyme (Mg^{2+} -ATPase) throughout the gradient is somewhat puzzling. This is probably an artifact of the homogenization procedure, which undoubtedly breaks the plasma membranes into various-sized fragments. Such fragments might well bind to granules and/or various proteins, forming aggregates that would spread widely throughout the gradient. At any rate, it is quite clear that the sedimentation characteristics of the oxidase particles are very unlike that of the Mg^{2+} -ATPase; the discrete peak of oxidase activity is similar to the distribution pattern of other granules. Thus it seems highly unlikely that the oxidase(s) is (are) located in the plasma membrane.

The fact that NADH and NADPH oxidase co-sediment in the gradients may indicate that two separate enzymes are contained within the same particle. Alternatively, it may be that a single enzyme exists in the cell, which can utilize either reduced nicotinamide nucleotide as substrate. The present experiments cannot distinguish between these two possibilities.

The nature of the oxidase-containing granule is of considerable interest. It appears to be larger and more dense than the azurophil granule and might represent a new class of granule within the leucocyte. In this regard, Breton-Gorius *et al.* (1975) used electron microscopy to study the neutrophilic granules of two patients with a complete deficiency of myeloperoxidase. No peroxidase reaction was observed after incubation in the usual medium of Graham & Karnovsky (1966). However, in alkaline medium in

the presence of cyanide, a peroxidase activity appeared in certain large elongated granules; Breton-Gorius *et al.* (1975) suggested that this might represent a peroxidative activity of NADPH oxidase. It is tempting to speculate that the particles containing the oxidase, which sediment at a density of 1.24 g/cm^3 in the sucrose gradient, correspond to these large granules described by Breton-Gorius *et al.* (1975). More experiments will be required to test this hypothesis.

Note Added in Proof

We have very recently discovered a report (Patriarca *et al.*, 1977) that is directly pertinent to the present paper. Patriarca *et al.* (1977) likewise used sucrose-gradient centrifugation and observed NADPH oxidase activity deep in the gradient, slightly lower than the region of myeloperoxidase activity, in accord with our present observations. No activity was associated with the plasma-membrane fraction as assayed by alkaline phosphatase activity; the distribution of NADH oxidase activity was not investigated.

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