The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils

Specific labelling of a component polypeptide of the oxidase

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NADPH-dependent superoxide production by the solubilized oxidase of neutrophils was inhibited 36% by diphenylene iodonium at a 1:1 stoichiometry with the enzyme flavoprotein content. Addition of diphenylene iodonium strongly inhibited the NADPH-dependent reduction of both FAD and cytochrome b_{-245} in steady-state kinetic experiments. Incubation of solubilized enzyme with diphenylene [125 I]iodonium resulted in the specific labelling of a polypeptide of M_r 45000. In the presence of NADPH the amount of label incorporated into the polypeptide was reduced. There was no difference in labelling between enzyme prepared from stimulated or unstimulated cells.

INTRODUCTION

Polymorphonuclear leukocytes (neutrophils) are responsible for the defence of the host against infection. A major weapon in their armoury is a membrane-bound oxidase that can be activated by a number of soluble or particulate stimuli. Stimulation of this oxidase system is responsible for the increased oxygen uptake of phagocytosis (the 'respiratory burst'), which is resistant to inhibitors of mitochondrial respiration such as cyanide and antimycin (Baldridge & Gerard, 1933; Rossi & Zatti, 1964; Babior, 1978). The electron donor for the oxidase is NADPH and the primary product is the superoxide anion radical ($O_2^{\cdot-}$), which can undergo a series of reactions to give H_2O_2 and the hydroxyl radical ($OH^{\cdot}$); these molecules may undergo further reactions to give other highly reactive species (fairly recently reviewed by Babior, 1984). These active oxygen species are toxic to micro-organisms and may be involved in tumour-cell killing (Hafeman & Lucas, 1979). Inappropriate stimulation of the cells may be responsible for the tissue damage in such conditions as rheumatoid arthritis and gout (Dieppe & Doherty, 1982). We have shown that this oxidase system contains both a b -type cytochrome with a very low oxidation-reduction potential ($E_{m,7.0} = -245$ mV; Cross *et al.*, 1981) sufficient to reduce oxygen to superoxide at a rapid rate, and a flavoprotein, which has non-covalently bound FAD as its prosthetic group (Light *et al.*, 1981; Cross *et al.*, 1982b; Gabig, 1983). By using a soluble preparation of the oxidase from pig neutrophils, we have analysed the kinetics of reduction under anaerobic conditions and found that the rate of cytochrome b_{-245} reduction is too slow to support the known rate of superoxide production (Cross *et al.*, 1984). However, we have recently been able to show that, under aerobic conditions, this anomaly disappears; by measurements of the extent of reduction of cytochrome b_{-245} in the aerobic steady state and by determination of the pseudo-first-order rate constant for reoxidation of reduced cytochrome b_{-245} , the specific rate of cytochrome reduction can be calculated (Cross *et al.*,

1985). This rate is almost identical with the measured rate of $O_2^{\cdot-}$ production. There is no reduction of cytochrome or flavoprotein in preparations from unstimulated cells. Three important conclusions can be derived from this work: (a) all electrons from NADPH pass through cytochrome b (there is no branch at the level of FAD); (b) oxygen is necessary for rapid electron transfer through the complex; and (c) the 'switch' for the activation of the oxidase is at the level of, or prior to, the flavoprotein. These results have led us to propose the scheme shown below:

Purification and partial characterization of the cytochrome component of the oxidase from both human and animal sources has been reported by several groups of workers, although with differing M_r values [68000–78000 (Harper *et al.*, 1984); 14000, 12000 and 11000 (Pember *et al.*, 1984); 127000 (Lutter *et al.*, 1985)], but characterization of the flavoprotein has been hampered by the difficulty in identifying a particular polypeptide as belonging to the flavoprotein. Here we report on the effects of a novel inhibitor of the oxidase, diphenylene iodonium (DPI, dibenziodolium, CAS [244-54-2]), and specific labelling of a polypeptide of M_r 45000 by diphenylene [125 I]iodonium ([125 I]DPI). A polypeptide with similar properties (M_r 44000–46000) is phosphorylated when the NADPH oxidase of neutrophils is stimulated (Gennaro *et al.*, 1985), and this polypeptide is missing or altered in a patient with the autosomal recessive form of chronic granulomatous disease, where there are reduced levels of flavoprotein (Segal *et al.*, 1985).

MATERIALS AND METHODS

Chemicals

Iodonium diphenyl (diphenyliodonium) and iodonium dithiophen (di-2-thienyl iodonium) were generously given by Dr. F. Earley, Department of Biochemistry,

Abbreviation used: DPI, diphenylene iodonium.

University of Southampton, Southampton SO9 3TU, U.K. DPI sulphate was synthesized as described by Collette *et al.* (1956). [^{125}I]DPI was synthesized as described by Gatley & Sherratt (1976). Na^{125}I was obtained from Amersham International, Amersham, Bucks., U.K. Solutions of DPI were standardized by using an ϵ_{284} value of $15.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Ragan & Bloxham, 1977).

Preparation of soluble oxidase

Neutrophils were isolated from pig blood, stimulated with phorbol myristate acetate and solubilized in detergent as described previously (Cross *et al.*, 1984).

Inhibition studies

Inhibition of the solubilized $\text{O}_2^{\cdot-}$ -generating oxidase was assayed either by adding small ($< 10 \mu\text{l}$) quantities of iodonium diphenyl (I), iodonium thiophen (II) or DPI (III) dissolved in 50% (v/v) ethanol to the $\text{O}_2^{\cdot-}$ assay

mixture or by incubation of small ($< 10 \mu\text{l}$) quantities of inhibitors with 1 ml aliquots of enzyme for 2 h or 24 h on ice before assay. Addition of $10 \mu\text{l}$ of 50% ethanol alone had no effect on superoxide production.

Radioactive labelling

Aliquots of enzyme were incubated for 10 min at 20°C in the presence of [^{125}I]DPI with or without 2 mM-NADPH and then used for electrophoresis without further treatment.

SDS/polyacrylamide-gel electrophoresis

Samples were solubilized in SDS sample buffer and separated by electrophoresis on 12%-(w/v)-polyacrylamide slab gels using the Laemmli (1970) system of buffers. If the gel was to be used for autoradiography, the gel was fixed in 20% (v/v) methanol for 2 h after electrophoresis, followed by 2 h in 20% methanol/5% glycerol (final concns.). The gel was then dried overnight on a glass plate between two sheets of cellophane securely clamped at the edges by bulldog clips. The dried gels were autoradiographed against Fuji-Rx film for 3 days. After autoradiography the gels were rehydrated and stained in the conventional manner.

Spectroscopy

Spectra were recorded on a rapid-scanning recording spectrophotometer as described previously (Cross *et al.*, 1984). Spectra of the steady-aerobic-state level of reduction of redox components in the presence of NADPH were recorded as described previously (Cross *et al.*, 1985) at 20°C .

Determination of $\text{O}_2^{\cdot-}$ production

NADPH-dependent superoxide production was determined by the superoxide-dismutase-sensitive rate of reduction of cytochrome *c* as described previously (Cross *et al.*, 1982a). The incubation mixture consisted of 2 mM-NADPH, $100 \mu\text{M}$ -horse heart cytochrome *c* (Sigma type III) in 1 ml of 50 mM-triethanolamine/HCl, pH 8.0, at 20°C . The rate of cytochrome *c* reduction was determined in the absence or presence of $100 \mu\text{g}$ of superoxide dismutase ($3000 \text{ units} \cdot \text{mg}^{-1}$).

Determination of flavin and cytochrome b_{-245} content

The reduction states of flavin and cytochrome were assayed in oxidized-minus-reduced difference spectra using $\Delta\epsilon_{450-500} = 11.3 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ and $\Delta\epsilon_{559-540} = 21.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ respectively (Cross *et al.*, 1984).

RESULTS

Inhibition of $\text{O}_2^{\cdot-}$ production from the solubilized enzyme by DPI

DPI caused rapid inhibition of enzyme activity when added to the buffer during the superoxide assay (Fig. 1). At concentrations above $10 \mu\text{M}$, inhibition was apparently complete within the mixing time (approx. 2 s); at lower concentrations the inhibition became increasingly time-dependent (Fig. 2). With respect to this time-dependence, the inhibition of the neutrophil oxidase closely resembles the inhibition of NADH: ubiquinone reductase by DPI (Ragan & Bloxham, 1977). Owing to the spontaneous decay of enzyme activity ($t_{1/2} = 36 \text{ s}$ during enzyme turnover in the $\text{O}_2^{\cdot-}$ assay), measurement of enzyme inhibition at very low concentrations of the inhibitor becomes increasingly difficult, and therefore the enzyme was preincubated on ice for 1 h in the presence of low concentrations of DPI. After 1 h incubation of DPI with the solubilized oxidase at 0°C , inhibition was still largely time-dependent when the enzyme/inhibitor mixture was added to the assay (result not shown), but after increasing the incubation time to 24 h (at 0°C) inactivation of the enzyme had occurred before addition to the assay. Under these conditions 36% inhibition of the oxidase activity is observed at concentrations of DPI at which the stoichiometry of the inhibitor/FAD content of the enzyme is 1:1 (Fig. 3). Addition of 100-fold excess of DPI does not cause total inhibition, approx. 15% of the activity remaining at this concentration. At higher concentrations (300–600-fold excess) no residual activity could be detected.

Addition of $50 \mu\text{M}$ -DPI to phorbol myristate acetate-stimulated intact neutrophils (Fig. 1) caused marked inhibition of superoxide generation, indicating that the cells are permeable to the inhibitor (in keeping with the lipid solubility of DPI) or its site of action is exposed to the external medium.

Fig. 1. Effect of iodonium compounds on superoxide production by solubilized oxidase and intact neutrophils

Superoxide production was determined by the superoxide-dismutase-sensitive rate of reduction of cytochrome *c* as described in the Materials and methods section. In (a)–(c), 10 µl of enzyme solution is equivalent to 50 µg of protein. In (d) 10^6 cells were used in place of enzyme. Abbreviation used: PMA, phorbol myristate acetate.

Inhibition of NADPH-dependent $O_2^{\cdot-}$ generation by other iodonium compounds

The superoxide-generating enzyme was also sensitive to two other iodonium compounds. Iodonium thiophene and iodonium diphenyl both caused 90% inhibition of activity at 5 µM. The sensitivity shown towards the former compound is similar to that found for NADH:ubiquinone reductase (50% inhibition at 2.5 µM-iodonium thiophen) (Ragan & Bloxham, 1977), but the neutrophil enzyme was markedly more sensitive to the latter compound than was the mitochondrial enzyme, which required 88 µM-iodonium diphenyl to give 50% inhibition.

Inhibition of the steady-state reduction of the redox components of the solubilized oxidase

In order to identify the site of action of DPI on the neutrophil superoxide-generating system, the effect of the inhibitor on the aerobic-steady-state level of reduction of the cytochrome b_{-245} (at 559 nm) and flavoprotein (at 450 nm) was determined. In the presence of 2 mM-NADPH, 13.6% of the cytochrome b_{-245} and 52% of the

flavin was reduced (Fig. 4); this corresponds to a specific rate of cytochrome b_{-245} reduction of $19.9 \text{ s}^{-1} \cdot \text{mol}$ of cytochrome b^{-1} (using the pseudo-first-order rate constant for cytochrome b_{-245} reoxidation, $k = 147 \text{ s}^{-1}$; Cross *et al.*, 1985). The specific rate of $O_2^{\cdot-}$ production was $19.4 \text{ s}^{-1} \cdot \text{mol}$ of cytochrome b^{-1} , in keeping with our previous findings (Cross *et al.*, 1985) that all electrons transferred from NADPH to oxygen pass through cytochrome b_{-245} . In the presence of 2 µM-DPI the reduction of both cytochrome b_{-245} and flavoprotein by NADPH were virtually abolished (Fig. 4) and $O_2^{\cdot-}$ production was inhibited to the extent of 90%. That both cytochrome and flavoprotein reductions are inhibited strongly suggests that the site of action of DPI is at the level of (or possibly before) the flavoprotein. In the case of inhibitors of the cytochrome component (such as *p*-chloromercuribenzoate) there is strong inhibition of cytochrome reduction, but much less pronounced inhibition of the flavoprotein reduction (Cross *et al.*, 1984).

Binding of DPI to a constituent polypeptide of the solubilized enzyme

The extreme sensitivity of the solubilized superoxide-generating system to DPI and the similarity of the pattern

Fig. 2. Time-dependence of the inhibition of the solubilized superoxide-generating oxidase by DPI

Superoxide production was determined by the superoxide-dismutase-sensitive rate of reduction of cytochrome *c* as described in the Materials and methods section. Inhibitor was added to the assay mixture at the required concentration and the reaction initiated by the addition of the solubilized oxidase. The activities are expressed as the logarithm of the percentage of the initial rate in the absence of the inhibitor, corrected for the spontaneous decay of activity. The following concentrations of DPI were used; $\blacktriangle$, $0.2 \mu\text{M}$; $\triangle$, $0.4 \mu\text{M}$; $\blacksquare$, $0.79 \mu\text{M}$; $\square$, $1.58 \mu\text{M}$; $\blacktriangledown$, $3.95 \mu\text{M}$; $\triangledown$, $7.9 \mu\text{M}$. The cytochrome b_{-245} and flavin concentrations were 1.87 nM and 2.44 nM respectively. The protein concentration was $4.8 \mu\text{g} \cdot \text{ml}^{-1}$.

of inhibition to that of the inhibition of NADH: ubiquinone reductase described by Ragan & Bloxham (1977) suggested that a radiolabelled analogue could be used to label specifically a component of the neutrophil oxidase. After incubation of aliquots of the solubilized enzyme with $[^{125}\text{I}]\text{DPI}$ at 20°C for 10 min followed by SDS/polyacrylamide-gel electrophoresis and autoradiography, significant radioactivity was consistently associated with a polypeptide of $M_r 45000$ in preparations of enzyme from both stimulated and unstimulated cells; occasionally very minor labelling could be seen of polypeptides of $M_r 70000$, 40000 and 36000 , particularly at high ratios of $[^{125}\text{I}]\text{DPI}$ to enzyme (Fig. 5). Other radioactivity was associated with the tracking-dye front, presumably attached to lipid or associated with SDS anions (Ragan & Bloxham, 1977). To prevent dissociation of the label during staining and destaining procedures, it was found advantageous to omit acetic acid from the buffers. If crude neutrophil membranes (prepared by centrifugation of neutrophil homogenates at $100000 g$ for 1 h) were used in place of solubilized enzyme, a polypeptide of $M_r 22000$ – 23000 was also observed. This

presumably was the polypeptide of NADH: ubiquinone reductase which was labelled by $[^{125}\text{I}]\text{DPI}$ originally described by Ragan & Bloxham (1977).

The effect of NADPH on the labelling of the $45000\text{-}M_r$ polypeptide of the NADPH oxidase

The effect of NADPH on the incorporation of radioactivity into the $45000\text{-}M_r$ polypeptide was investigated. Portions of enzyme were incubated at 20°C in the presence or absence of 2 mM -NADPH and samples were removed for electrophoresis as described in the Materials and methods section. There was significantly less incorporation of radioactivity into the enzyme in the presence of NADPH (result not shown), indicating that the binding of NADPH to the enzyme may sterically mask or compete for the binding site for DPI, or may cause the enzyme to undergo a conformational change which decreases the binding of the inhibitor. At the concentration of DPI used in this experiment ($10 \mu\text{M}$) there was no apparent time-dependence in the incorporation of radioactivity.

DISCUSSION

Although the respiratory-burst oxidase has for some years been known to contain a flavoprotein, until the present time it has not been possible to identify a component polypeptide. The present results strongly suggest that the flavoprotein contains a polypeptide, of $M_r 45000$, which may be specifically labelled by $[^{125}\text{I}]\text{DPI}$, forming a complex that is stable to electrophoresis. It is very probable that this is the same polypeptide which is phosphorylated when neutrophils are activated (Gennaro *et al.*, 1985) and which is missing from a patient with the autosomal recessive form of chronic granulomatous disease where the flavoprotein is altered or absent (Segal *et al.*, 1985). The very low concentrations of DPI

Fig. 3. Effect of a 24 h incubation of the superoxide-generating oxidase with DPI

Portions of the superoxide-generating oxidase were incubated for 24 h on ice and assayed for residual oxidase activity as described in the Materials and methods section. The cytochrome b_{-245} and flavin contents were $347 \text{ pmol} \cdot \text{ml}^{-1}$ and $354 \text{ pmol} \cdot \text{ml}^{-1}$ respectively. The protein concentration was $0.8 \text{ mg} \cdot \text{ml}^{-1}$.

Fig. 4. Effect of DPI on the aerobic steady-state reduction of cytochrome b_{-245} and flavin by NADPH in the solubilized superoxide-generating oxidase

Spectra were recorded as described in the Materials and methods section. a, After a spectrum of the oxidized enzyme had been recorded, NADPH was added to a final concentration of 2 mM, the spectrum recorded within 1 min, and the oxidized spectrum subtracted. The absorbance scale of the spectrum has been expanded 2-fold for clarity. b, Complete reduction of the enzyme after the addition of a few crystals of sodium dithionite to a. c and d, Spectra were recorded as in a, except that 2 μ M-DPI was present throughout. The absorbance scale of c has been expanded 2-fold for clarity. The extent of reduction of the flavoprotein and cytochrome b_{-245} components were estimated as described in the Materials and methods section.

Fig. 5. Labelling of polypeptides by [^{125}I]DPI

Labelling of polypeptides by [^{125}I]DPI, autoradiography and protein staining were performed as described in the Materials and methods section. (a) Autoradiograph of an SDS/polyacrylamide gel of solubilized enzyme incubated with [^{125}I]DPI which was present in a 5-fold molar excess with respect to the flavin content of the preparation. (b) and (c), The same gel stained for protein with Coomassie Blue. (b) M_r markers: bovine albumin, 66000; egg albumin, 45000; glyceraldehyde-3-phosphate dehydrogenase, 36000; carbonic anhydrase, 29000; trypsinogen, 24000; trypsin inhibitor, 20100; and lactalbumin, 14200. (c) Solubilized enzyme.

required to inhibit the enzyme also almost completely abolish the reduction of both flavoprotein FAD and cytochrome b_{-245} , which strongly suggests that the binding of DPI to the flavoprotein is responsible for the inhibition of enzyme activity. These same low concentrations of inhibitor also label a single polypeptide. Since the labelling of the polypeptide is suppressed in the presence of NADPH, it is likely that the site of attachment is close to the NADPH-binding site. As there is apparently no difference in reactivity towards the enzyme prepared from resting or activated cells, it can be inferred that activation of the enzyme does not result from an alteration in the ability to bind NADPH, since this would be expected to cause a difference in the intensity of labelling, as found in the labelling experiment in the presence or absence of NADPH. In many respects the inhibition of the neutrophil oxidase is strikingly similar to the inhibition of NADH:ubiquinone reductase by DPI described by Ragan & Bloxham (1977). In particular, the disproportionately large increase in inhibitor concentration required to inhibit the residual

30% of activity (Fig. 3) and the time-dependence of inhibition at low concentrations of inhibitor (Fig. 2) suggest very similar mechanisms of inhibition. Unfortunately we cannot distinguish between second-order labelling (at higher DPI concentrations) and affinity labelling at the present time. The neutrophil superoxide-generating system does differ, however, in being more sensitive to iodonium diphenyl than is the mitochondrial enzyme. The chemical nature of the covalent adduct formed between DPI and the protein is not known. In view of the acid-lability of the labelling, Ragan & Bloxham (1977) suggested that a carboxyl or imidazole derivative would be more probable than the more stable mercaptan or amine derivatives.

The ability to locate a subcellular or column fraction containing the flavoprotein moiety of the oxidase by means of detection of radioactivity should prove a useful tool in both biochemical and cell-biological studies of the neutrophil oxidase. In view of the probable involvement of the neutrophil superoxide-generating system in inflammatory processes and its extreme sensitivity to DPI, it is possible that compounds of this type (without the undesirable hypoglycaemic properties of DPI; Bloxham, 1979) may prove to have useful anti-inflammatory properties.

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