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Identification of the haem-binding subunit of cytochrome b_{-245}

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Cytochrome b_{-245} from neutrophil plasma membranes contains two types of subunit with apparent molecular masses from gel electrophoresis in the presence of SDS of 23 kDa and 76–92 kDa. Radiation-inactivation analysis revealed a single-exponential decay process for the visible absorption of the haem chromophore in the membrane, corresponding to a molecular mass of 21 ± 5 kDa for the haem-containing polypeptide chain. Sedimentation equilibrium of the cytochrome solubilized by the detergent Triton N101 showed that the protein was polydisperse, with a molecular mass of approx. 350 kDa for the smallest detectable species. In another detergent, n-octyl β-*O*-glucopyranoside (octyl glucoside), the molecular mass of the haem-containing particle was found to be 20–30 kDa. Thus the quaternary structure of the protein breaks down in this detergent. The haem group is inferred to be attached to the smaller subunit.

INTRODUCTION

The efficient destruction of microbes by the phagocytic cells, neutrophils, monocytes, macrophages and eosinophils, depends upon the generation of superoxide radicals through the action of an oxidase system. Chronic granulomatous disease, which is characterized by a profound predisposition to infection, results from a defect in this system. The oxidase is located in the phagocytic vacuole membrane and comprises an electron-transport chain that translocates electrons from cytosolic NADPH into the vacuole, where superoxide radicals are formed (see Segal, 1989).

Cytochrome b_{-245}, which binds CO (Cross *et al.*, 1981, 1982) and is thought to be the terminal component of the oxidative cascade, is the only one of these proteins to have been unambiguously identified. It is an oligomer, possibly a dimer, made up of two types of subunit (Segal, 1987; Parkos *et al.*, 1987), denoted α and β, with molecular masses derived from the cDNA sequences of their respective genes of 21 kDa (Parkos *et al.*, 1988*a*) and 65 kDa (Dinauer *et al.*, 1987; Teahan *et al.*, 1987). There has been uncertainty about which of these subunits contains the haem group, because conditions that cause dissociation of the quaternary structure have in general been found to denature the proteins with consequent loss of the haem. In most cases of X-chromosome-linked chronic granulomatous disease, in which cytochrome *b* cannot be detected spectroscopically, the genetic lesion is in the β-chain gene (Teahan *et al.*, 1987; Dinauer *et al.*, 1987), but neither protein appears in the membranes (Segal, 1987).

The associated state of the cytochrome in detergent solutions has been uncertain because of extensive glycosylation of the β-chain (Harper *et al.*, 1985), which renders SDS/polyacrylamide-gel electrophoresis highly unreliable, and because of the unknown extent of detergent and possibly phospholipid binding. This creates problems in the interpretation of sedimentation data in the presence of a mixed solvent system (sucrose/water) and of Stokes radii, derived from gel filtration (Tanford & Reynolds, 1976). We have attempted to study the association state of the cytochrome by sedimentation equilibrium in two detergent systems of interest, and have made independent estimations of the size of the haem-bearing subunit by radiation-inactivation target analysis (Kempner & Schlegel, 1979). We find that the haem group is associated with the α-chain. Our conclusion agrees with that of a recent study by Yamaguchi *et al.* (1989).

METHODS

Purification of cytochrome *b*

Cytochrome b_{-245} was purified from human neutrophils obtained from buffy-coat residues as described previously (Harper *et al.*, 1984). Briefly, pelleted organelles from a post-nuclear supernatant were extracted in buffer A [0.1 M-Tris/acetate buffer, pH 7.4, containing 0.1 M-KCl, 20% (v/v) glycerol, 1 mM-dithiothreitol, 1 mM-EDTA, 30 μM butylated hydroxytoluene, 1 mM-phenylmethanesulphonyl fluoride and 100 kallikrein-inactivating units of Trasylol (Bayer, Haywards Heath, West Sussex, U.K.)/ml] containing 0.5% cholate before extraction of the cytochrome into buffer A containing 1% (w/v) Triton N101. The extract was passed through DEAE-Sepharose and CM-Sepharose before adsorption on heparin–agarose, from which it was eluted with a linear NaCl gradient. In order to concentrate the cytochrome or change detergents, the peak of eluted cytochrome was diluted 1 in 10 with ice-cold water and reapplied to a small (1 cm × 2 cm) column of heparin–agarose. It was then equilibrated with the desired buffer before elution with the same buffer containing 0.5 M-NaCl. The cytochrome used in sedimentation-equilibrium studies had a specific activity of 7.0 nmol/mg [taking $\epsilon_{559} - \epsilon_{540} = 21.6\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ (Cross *et al.*, 1982) and protein assayed with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.)]. Unless specified, all chemicals were from Sigma Chemical Co.

Purification of neutrophil membranes

Membranes were isolated from neutrophils described above by centrifugation on discontinuous sucrose gradi-

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ents (Segal & Jones, 1979). They contained the cytochrome *b* in a concentration of about 400 pmol/mg of protein.

Sedimentation-equilibrium studies

Sedimentation studies were performed in the Beckman model E analytical ultracentrifuge equipped with scanner optics. To obtain the distribution of the haem-associated protein, a wavelength of 413 nm was selected. Satisfactory protein concentration gradients were achieved at rotor speeds of 4000 rev./min in Triton N101 (10 mg/ml) and at 10000 rev./min in octyl glucoside (12 mg/ml). Liquid column heights of 2–3 mm were used, and measurements were made after 36 h of sedimentation at about 15 °C. To eliminate the detergent contribution to the partial specific volume of the sedimenting particle, the ^2H_2O masking procedure of Reynolds & Tanford (1976) was employed. The apparent reduced molecular mass $M[1-v_P\rho)+\delta_D(1-v_D\rho)]$ [in which M (in Da) is the molecular mass of the non-complexed protein, δ_D is the weight of detergent bound (g/g of protein), v_P and v_D are the partial specific volumes of the protein and detergent and ρ is the solvent density] was determined in buffer (0.2 M-NaCl) prepared in a series of mixtures of ^1H_2O and ^2H_2O. The function is plotted against ρ, and its value at the point at which $\rho^{-1} = v_D$ gives the reduced molecular mass of the protein without the bound detergent. The value of v_P in octyl glucose was calculated by using the amino acid residue contributions tabulated by Perkins (1986) from the derived amino acid composition (Parkos *et al.*, 1988*a*).

Fluorescence spectroscopy

Fluorescence spectra were measured in a Perkin–Elmer MPF32 instrument, with excitation at 305 nm to minimize any contribution from the Triton X-100.

Radiation-inactivation analysis

Samples (0.3 ml) of purified neutrophil membranes (5 mg of protein) in 50 mM-Hepes/NaOH buffer, pH 7.5, containing malate dehydrogenase (pig heart cytoplasmic; EC 1.1.1.37) (1.0 μg/ml), bovine serum albumin (2 mg/ml), NaCl (15 mM), glycerol (20%, v/v) and silicone antifoam (10 μl/ml) were prepared in quartz glass tubes, flushed with N_2 gas for 30 min and then frozen at 77 K.

Irradiation of frozen samples was carried out at 77 K with a 16 MeV electron beam (MEL SL 75/20 linear accelerator, at Addenbrooke's Hospital, Cambridge, U.K.) as described previously (Nugent, 1986; Nugent & Bendall, 1987). The dose rate was 2 Mrad/min, checked by Perspex [poly(methyl methacrylate)] dosimetry. Doses were accumulated in 15 Mrad steps. After treatment the tubes were flushed with N_2 to remove O_2 and O_3 before thawing and assay of samples.

Malate dehydrogenase activity was assayed as described previously (Nugent, 1986). The cytochrome was measured by the height of the Soret peak of absorption in reduced-minus-oxidized difference spectra by scanning between 600 and 400 nm in a Pye–Unicam SP.8-200 spectrophotometer after the addition of solid $Na_2S_2O_4$. Activities are expressed as percentages of the control value and target analysis was performed as described by Nugent & Atkinson (1984). If activity is lost by a single rate process, a linear plot of dose versus log(activity) is obtained. The slope of this plot is related to the molecular mass by using a standard enzyme of known molecular mass (Nugent, 1986). Regression lines were checked for error by a plot of standard residuals versus dose. Errors are expressed as standard deviations.

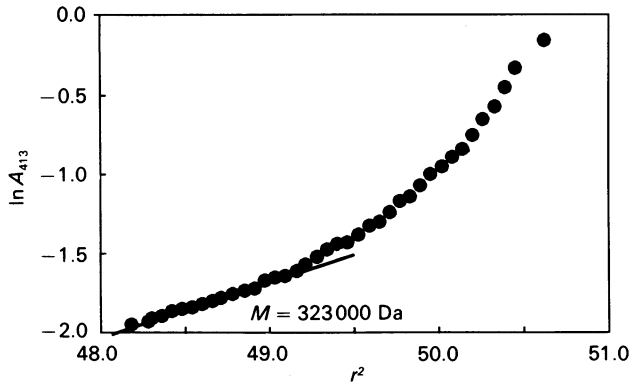

Fig. 1. Sedimentation-equilibrium distribution of cytochrome b_{-245} in buffer containing Triton N101 and 71% ^2H_2O with the use of scanner optics at a wavelength of 413 nm

The rotor speed was 4000 rev./min. At the ^2H_2O concentration used here the detergent contribution to the partial specific volume is expected to vanish.

RESULTS AND DISCUSSION

Equilibrium centrifugation

Sedimentation-equilibrium distributions were determined in the presence of the detergent Triton N101, in which the α- and β-subunits evidently remain associated. In gel filtration and affinity chromatography on heparin–agarose, the elution profiles of haem and both subunits are the same, and in a similar detergent, Triton X-100, the subunits can be cross-linked to each other by a heterobifunctional reagent (Parkos *et al.*, 1988*b*). Thus we may take it that the haem distribution in sedimentation equilibrium reflects that of the total protein.

Fig. 1 shows such an experiment at a proportion of ^2H_2O in the solvent of 71%, at which the solvent density matches that of the detergent (Tanford & Reynolds, 1976). The protein is seen to be highly polydisperse.

The molecular-mass distribution in such a system cannot be explicitly determined, unless an assumption is made about the nature of the association (e.g. isodesmic association of a protomer). However, an analysis of the slope of the equilibrium plot from the top to the bottom of the fluid column (Roark & Yphantis, 1969) reveals that through the top 25–30% of the column height the molecular mass is constant, and must thus correspond to the smallest molecular species present. Its reduced molecular mass, $\lambda \equiv M(1-\phi'\rho)$, where ϕ' is the true volume increment per g of dry protein, is 87 kDa. The value of ϕ is in some doubt because of uncertainty in the extent of glycosylation of the β-subunit and the composition of the carbohydrate (Harper *et al.*, 1985; Parkos *et al.*, 1987). From the sequence-derived values of 21 kDa and 65 kDa for the protein molecular masses of the α- and β-chains respectively, a partial specific volume for the total protein of 0.74 ml/g and a typical value of 0.64 ml/g (Perkins, 1986) for the carbohydrate, we obtain a notional value for ϕ' of 0.70, and thence a minimum molecular mass of about 350 kDa. Thus in this detergent the

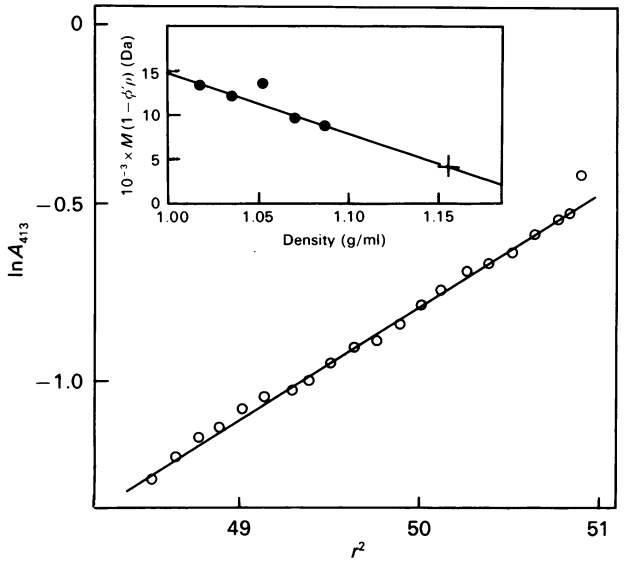

Fig. 2. Typical sedimentation equilibrium of the haem-containing constituent of cytochrome b_{-245} in the presence of octyl glucoside

Scanner optics were used at a wavelength of 413 nm and the rotor speed was 10000 rev./min. The proportion of ^2H_2O in the buffer in this case was 60%. Inset: extrapolation of reduced molecular mass to the density corresponding to masking of detergent contribution to the partial specific volume, as indicated; the least-squares line is shown, but the deviant point has been disregarded in the calculation.

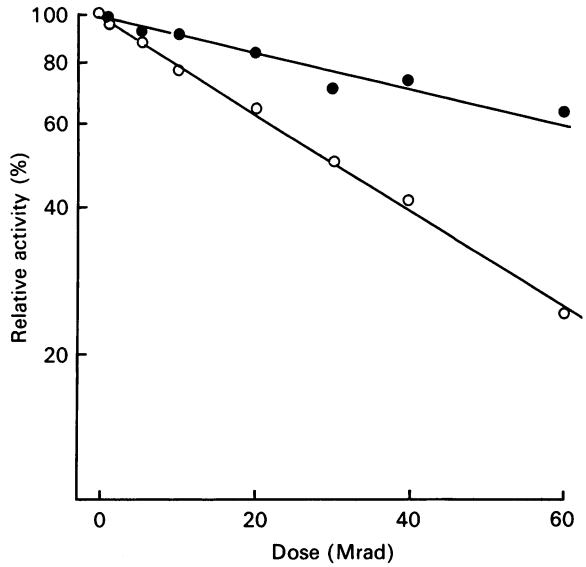

Fig. 3. Target-size analysis of malate dehydrogenase (○) and the Soret band of cytochrome b_{-245} (●)

The slope for malate dehydrogenase (72 kDa) $= -9.9 \times 10^{-3} \pm 0.3 \times 10^{-3}$. The slope for cytochrome b_{-245} $= -2.9 \times 10^{-3} \pm 0.5 \times 10^{-3}$. This gives a molecular mass of 21 ± 5 kDa for the haem-binding subunit of this cytochrome.

cytochrome exists as oligomers in the form $\alpha_n\beta_n$, with $n > 2$ or possibly 3.

These results differ from that of the cytochrome complex in Triton X-100, determined as 100 kDa by Parkos *et al.* (1988*b*) by sucrose-gradient sedimentation in $^1H_2O/^2H_2O$ combined with a Stokes radius from gel filtration. The problems generated by the mixed solvent system of water and sucrose that they have used to determine (or eliminate) the partial specific volume of the bound detergent have been analysed by Tanford & Reynolds (1976).

Sedimentation equilibrium of the cytochrome in octyl glucoside, which has a high critical micelle concentration, showed that the haem-containing entity in this case is essentially monodisperse (Fig. 2). Because of the low partial specific volume of octyl glucoside the determination of $M(1-\phi'\rho)$ requires extrapolation (Fig. 2 inset), which greatly limits the precision of the estimation. Depending upon the value of the partial specific volume of octyl glucoside selected from the literature (we used the value of 1.14; Lässer & Elias, 1972), the inferred molecular mass is 22 kDa or 29 ± 3 kDa (mean $\pm$ S.E.M.), with 95% confidence limits of ± 10 kDa. It thus appears that in this detergent the haem group is attached to the smaller (α-)subunit, which has dissociated from its partner.

The slope of the linear plot of reduced molecular mass against solvent density is related to the amount of detergent bound to protein (Reynolds & Tanford, 1976) by the relation $-d\lambda/d\rho = M(v_P + \delta_D v_D)$. Applying this to the data of Fig. 2 (inset), we obtain a value for bound detergent (δ_D) of 1.8 g/g.

In some sedimentation-equilibrium distributions a greater degree of upward curvature towards the bottom of the liquid column was seen, reflecting either incomplete dissociation or aggregation of the subunits. Nevertheless, the results strongly suggest that the α- and β-chains are largely dissociated from each other in octyl glucoside, the α-chains remaining monomeric.

To observe the dissociation of the α- and β-subunits by octyl glucoside, an excess of octyl glucoside (20 mg/ml) was added to a solution of the protein in the buffer, containing 0.1% Triton X-100, at 24 °C. The fluorescence of the protein showed a typical tryptophan emission maximum at 340 nm, characteristic of 'red' tryptophan residues, in an apolar environment in the protein interior. The fluorescence of tryptophan residues close enough to the haem group is expected to be quenched; thus no emission is expected from the haem-containing subunit, and appreciable quenching of that from the other subunit is likely when the protein is in its associated state. On dissociation of the quaternary structure an increase in fluorescence intensity should occur. This is indeed seen, although the effect is not large. Over 3 h after addition of the octyl glucoside the fluorescence at 340 nm increased by some 10%, and after 15 h the increase was 20%. This is consistent with a slow dissociation process in octyl glucoside. Attempts were therefore made to separate the chains by eluting the cytochrome from heparin–agarose with a salt gradient or by gel filtration on Superose 12, both in the presence of octyl glucoside (10 mg/ml). These experiments were unsuccessful, irrespective of the temperature between 4 °C and 25 °C, of NaCl concentrations up to 1 M and of incubation time. No significant fractionation of subunits could be demonstrated, and at high ionic strengths the absorption spectrum changed towards that characteristic of free haem. We cannot account for the seeming discrepancy between these observations and

the sedimentation-equilibrium results, nor can we totally exclude the possibility of artifacts, due for example to proteolysis during the extended periods of the sedimentation experiments.

Nevertheless, after completion of these studies, a report by Yamaguchi *et al.* (1989) appeared that concluded that the subunits of the cytochrome were dissociated in the presence of octyl glucoside and that the haem group was attached to the smaller subunit. However, it is not self-evident that the cytochrome b_{-558} of Yamaguchi *et al.* (1989) is identical with cytochrome b_{-245}, as their protein comprised only a small proportion of the total cytochrome *b* in the neutrophil preparation and was obtained in 4% yield on the basis of cytochrome haem absorption. The chain length of the α-subunit is inconclusive evidence because many cytochrome *b* species have a molecular mass of about 20 kDa (Lemberg & Barrett, 1973). Although the spectral and CO-binding properties resembled those of cytochrome b_{-245}, the amino acid composition was not close to that predicted by the sequence of the cDNA for the cytochrome b_{-245} α-chain (Parkos *et al.*, 1988*a*).

Radiation inactivation

To obtain independent evidence of the identity of the haem-binding subunit, radiation-inactivation measurements were made on the cytochrome *in situ* in the neutrophil membrane, its absorption spectrum being used as the marker for inactivation.

The intensity of the Soret absorption maximum of the cytochrome *b* and the malate dehydrogenase standard both declined according to a single-exponential function of radiation dose (Fig. 3). The target size for the haem-binding structure of the cytochrome was calculated to be 21 ± 5 kDa, exactly that of the α-subunit predicted from the cDNA nucleotide sequence (Parkos *et al.*, 1988*a*). The slope for the inactivation of malate dehydrogenase was similar to that of earlier studies (Nugent, 1986; Nugent & Bendall, 1987).

We conclude that the α-chain is the haemoprotein. This raises the question as to the role of the larger β-subunit, which could play a structural role in the maintenance of the haemoprotein in the membrane and its integration with other components of the electron-transport chain (Heyworth *et al.*, 1989).

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