

Comparison of p25 presequence peptide and melittin

Red blood cell haemolysis and band 3 aggregation

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The 25 residue presequence (p25) for subunit IV of yeast cytochrome oxidase has previously been shown to possess structural and behavioural characteristics in common with the bee venom polypeptide, melittin. The present study extends the results of leakage experiments on model-membrane systems to the haemolysis of human erythrocytes, which both peptides are shown to accomplish in a manner sensitive to membrane potential. In addition, the laser flash-induced transient dichroism technique for measuring protein rotational diffusion has been used to show that both peptides aggregate band 3, the major integral membrane protein of the erythrocyte. Aggregation cannot be reversed by high ionic strength; this serves to differentiate these peptides from other positively charged species such as polylysine that aggregate band 3 at low ionic strength. These results suggest that aggregation of membrane proteins may possibly prove to be a feature of the interaction of p25 signal peptide with mitochondrial membranes.

INTRODUCTION

The bee venom polypeptide melittin is known to cause significant disruption of the erythrocyte membrane [1,2]. Many other lytic agents have been compared with it, on the basis of possessing high surface activity and common secondary structural characteristics. Recently, it has been proposed that sequences directing proteins into the matrix space of mitochondria could also display such properties [3,4]. A particularly striking example is the 25 residue presequence (p25) for subunit IV of yeast cytochrome oxidase, a protein localized in the mitochondrial inner membrane. This peptide has recently been synthesized and shown to cause leakiness of small unilamellar vesicles in a potential-sensitive manner [3]. This resembles the finding that melittin-induced conductance in black lipid membranes is enhanced by a trans-negative polarization [5].

The mechanism of protein translocation following binding of the presequence to the membrane remains obscure. It does, however, appear likely that the presequence locally perturbs the membrane structure in a manner which facilitates the translocation step. A resemblance between presequences and agents such as melittin may therefore not be entirely coincidental. It would thus appear worthwhile to investigate further the extent of the similarities between p25 and melittin.

To pursue this aim we have used the erythrocyte membrane as a model system. Transient dichroism measurements [6] as well as electron microscopy [7; S. Hui, unpublished results] have previously shown that aggregation of band 3, the predominant integral protein of erythrocyte membranes [8], is one result of the presence of lytic concentrations of melittin. We have used here transient dichroism [9], to compare the ability of p25 with that of melittin to aggregate band 3, and also undertaken a comparative study of the haemolytic properties of the two peptides. The results obtained reveal a further correspondence between the properties of p25 and melittin.

EXPERIMENTAL

Materials

Melittin, low in phospholipase activity, prepared from whole bee venom was donated by Dr. R. C. Hider (University of Essex, Essex, U.K.). Its concentration was determined according to a molar absorption coefficient of 5600 cm^{-1} at 280 nm [10]. p25 presequence peptide was the generous gift of Dr. D. Roise (Biozentrum der Universität, Basel, Switzerland). Its concentration was determined by the Lowry method (using bovine serum albumin as a standard and a correction factor of 0.35). Stock peptide solutions were made up in water and stored frozen. Eosin-5-maleimide was from Molecular Probes Inc. Fresh blood (O⁺) obtained by venipuncture was used for haemolysis experiments. Ghosts for transient dichroism measurements were prepared from outdated blood (O⁺) from the local hospital.

Haemolysis

Fresh blood was washed three times in 5 mM-Hepes buffer (pH 7.5) containing 150 mM-KCl/1 mM-EDTA. The packed cells were resuspended in the same buffer to 1% (v/v). This red blood cell solution (0.5 ml) was rapidly combined with 0.5 ml of buffer containing varying peptide concentrations. After 7.5 min samples were centrifuged for 20 s in a microfuge, the supernatant was separated and its absorbance at 578 nm determined, relative to suitable controls. The percentage haemolysis was calculated by comparing each absorbance with that of a fully lysed sample. The procedure induced no haemolysis in the absence of peptide. In order to determine the time course of haemolysis, larger volumes were combined and 1 ml portions were taken from this bulk solution in time for the supernatant to be removed at the time indicated. Experiments were performed at room temperature unless otherwise indicated as problems due to loss of activity with time (possibly due to peptide sticking to the vessel wall) appear to be less severe than

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at 37 °C and the results are therefore correspondingly more reproducible. All experiments were done twice unless otherwise indicated; comparative experiments were all performed on the same red blood cell suspensions.

Potential dependence of haemolysis

These experiments were performed in a similar manner to the above. Cells were suspended to the same haematocrit (as judged by absorbance measurements on a fully lysed sample) in the same isotonic buffer, containing differing amounts of Na⁺ (0–0.15 M) in place of K⁺. A trans-membrane potential was induced by adding 3 μl of a 1.6 mM-valinomycin solution dissolved in dimethyl sulphoxide to 0.75 ml of red blood cell suspension 20 s before the mixing of 0.5 ml with an equal volume of peptide-containing buffer of the same composition. The samples were incubated for 3.5 min before spinning down.

The relative magnitudes of the induced potentials were checked using a potential-sensitive dye, diS-C₃(5), according to the method of Rink & Hladky [11]. The results corresponded well with the predicted variance in membrane potential as external K⁺ concentration changes, given by:

$$V_m = \frac{RT}{F} \log \frac{[K^+]_{\text{inside}}}{[K^+]_{\text{outside}}}$$

Measurement of band 3 rotation

The laser flash-induced transient dichroism technique has been described in detail elsewhere [8,12]. Briefly, band 3 of intact erythrocytes was selectively labelled by incubation with eosin-5-maleimide for 30 min at room temperature. Ghosts were prepared from these cells by hypotonic lysis and subsequent washing in 7.5 mM-Hepes buffer containing 1 mM-EDTA. Ghosts were added to peptide dispersed in buffer to a typical concentration of 0.8 mg of membrane protein/ml. The rotational diffusion

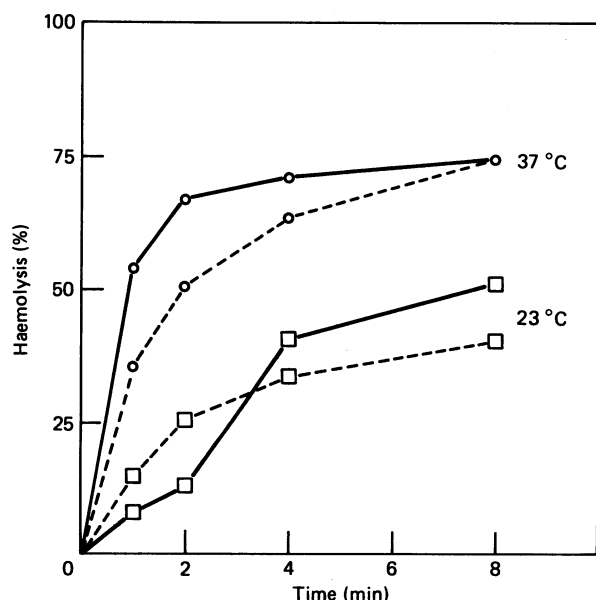


Fig. 1. Time course for the extent of induced haemolysis

A suspension of red blood cells [0.5% (v/v)] was incubated with 22 μg of p25 peptide/ml (solid lines) and 1 μg of melittin/ml (broken line) at 37 °C (○) and 23 °C (□).

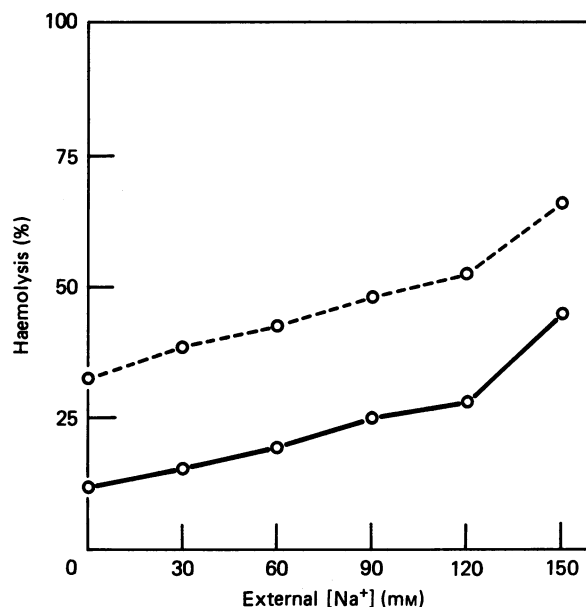


Fig. 2. Dependence of haemolysis on the trans-membrane potential

Extent of induced haemolysis after 3.5 min of incubation at 23 °C of an 0.5% (v/v) suspension of red blood cells with 26.25 μg of p25 peptide/ml (solid line) and 1.5 μg of melittin/ml (broken line). Before peptide addition a trans-membrane potential was induced by addition to the cell suspension of 3 μl of 1.6 mM-valinomycin, dissolved in dimethyl sulphoxide. The magnitude of the internally negative polarization increases with increasing external Na⁺ concentration (see the Experimental section).

of band 3 was measured by observing transient dichroism of ground-state depletion signals, arising from excitation of the probe into a triplet state by a linearly polarized light pulse from an Nd-YAG laser (JK Lasers, Rugby, U.K.). Excitation was at 532 nm and absorbance changes were recorded at 515 nm for light polarized parallel and perpendicular relative to the exciting flash. The signals were collected and averaged by a Datalab DL 102A signal averager.

Data were analysed by calculating the absorption anisotropy $r(t)$, given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)}$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ represent absorbance changes at time t , for light polarized parallel and perpendicular relative to the polarization of the exciting flash. The experimental decay curves were normally fitted to a double exponential equation.

$$r(t) = r_1 \exp(-t/T_1) + r_2 \exp(-t/T_2) + r_3$$

The interpretation of these decay curves is discussed in detail elsewhere [12].

RESULTS AND DISCUSSION

p25 was found to haemolyse red blood cells, though less efficiently than melittin (whose effects are already well-documented) [13,14]. Fig. 1 compares the time course for haemolysis for the two peptides and demonstrates that an approximately 20-fold-higher concentra-

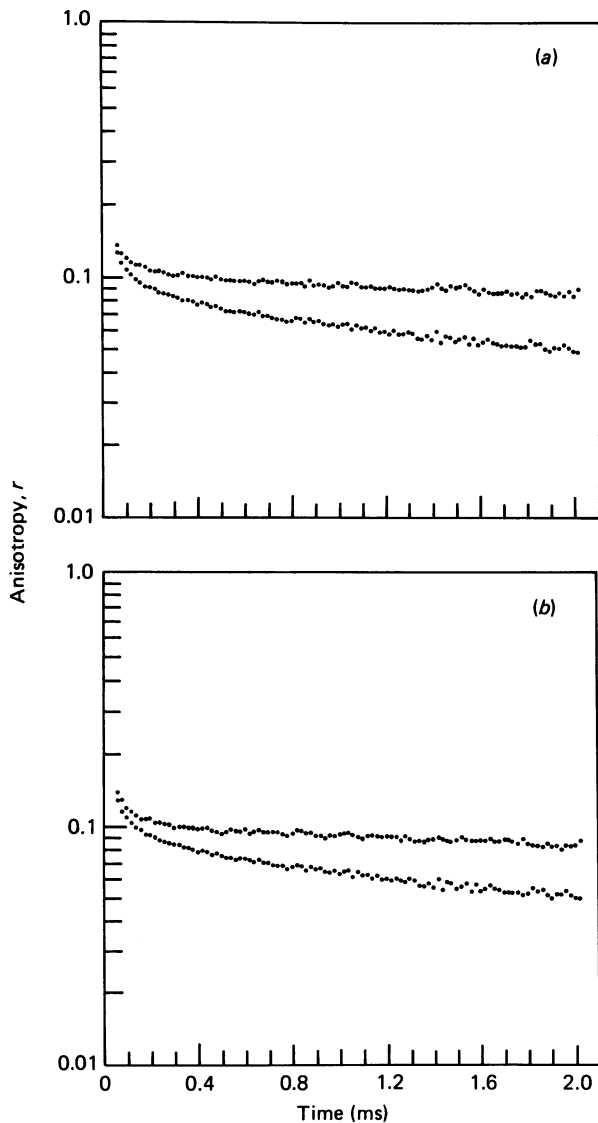


Fig. 3. Direct comparison of the capacities of p25 peptide and melittin to immobilize band 3

The lower curve on both diagrams is the anisotropy decay of eosin-labelled band 3 in control ghosts suspended in 7.5 mM-Hepes/1 mM-EDTA, pH 7.5 buffer, at 37 °C. The upper curve in each diagram is the anisotropy decay after addition of (a) 40 µg of melittin, (b) 38 µg of p25 peptide.

tion of p25 is required to produce the same effect as melittin. In both cases, haemoglobin release appears to follow a similar time course to that previously reported for melittin by De Grado *et al.* [13]. In both cases lysis occurs over a wide temperature range (4–37 °C), in contrast with the temperature sensitivity of some other haemolytic proteins [15,16]. More interestingly perhaps, the extent of haemolysis produced by both peptides, after trans-negative hyperpolarization of the erythrocyte membrane, is significantly increased (Fig. 2). This corresponds with both the previously reported increases in p25-induced leakage from liposomes [3] and the melittin-induced conductance across black lipid membranes, in the presence of a trans-negative membrane potential [5].

The transient dichroism data show p25 to be equally as capable as melittin of immobilizing band 3. Figs. 3 and

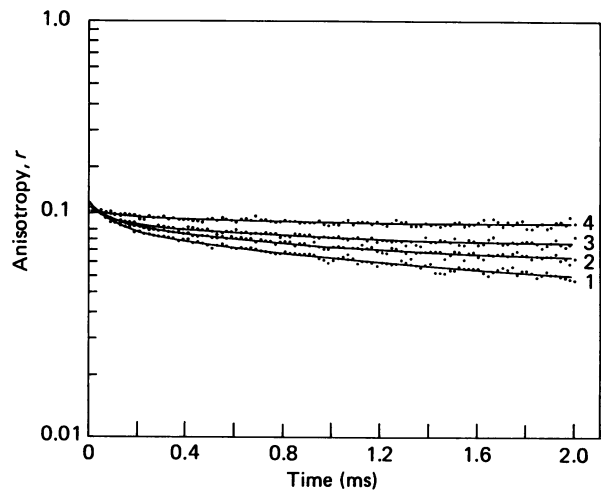
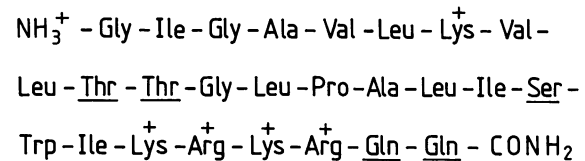


Fig. 4. Successive retardations of band 3 in erythrocyte ghosts with increasing p25 concentrations

Anisotropy decay curves after addition of (1) 0 µg, (2) 25 µg, (3) 40 µg, (4) 60 µg, of p25 peptide to ghosts suspended in 7.5 mM-Hepes/1 mM-EDTA, pH 7.5 buffer, at 37 °C.

Melittin



p25

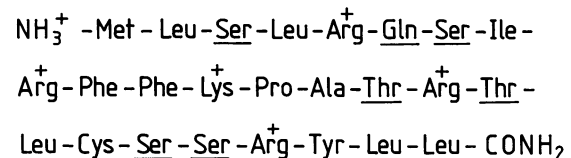


Fig. 5. Amino acid sequences of melittin and p25

Charges are as indicated and uncharged hydrophilic residues are underlined.

4 show absorption anisotropy decays arising from eosin-labelled band 3 in membranes subjected to similar concentrations of melittin and p25 (Fig. 3), and also of varying p25 concentration (Fig. 4). The anisotropy decay reflects the rotational motion of band 3 protein, which occurs only about an axis perpendicular to the plane of the membrane. The curves obtained upon successively increasing concentrations of either peptide demonstrate successive retardations in the rate of decay, thereby implying a decrease in rotational motion.

Such effects can be achieved by increasing the radius of the rotating species via protein aggregation. Freeze-fracture electron microscopic studies have supported this interpretation in the case of melittin: clustering of integral membrane particles is clearly visualized after

exposure of erythrocytes to relevant concentrations of the peptide [7; S. Hui, unpublished results]. Additionally, in both cases the immobilization was unaffected by incorporation of 0.15 M-NaCl into the 7.5 mM-Hepes buffer used for the transient dichroism measurements. This serves to distinguish melittin and p25 from other positively charged aggregating agents such as polylysine and bivalent metal ions which also immobilize band 3 but whose effects are diminished by increasing the ionic strength of the medium to this extent (M. J. Clague & R. J. Cherry, unpublished results).

Melittin and p25 protein have the amino acid sequences depicted in Fig. 5. Both are basic peptides which are thought to possess substantial α -helicity when bound to a membrane-like surface [3,17]. Helical wheel plots and for melittin, crystallographic determination [18], have shown the predicted regions of α -helix to be amphiphilic. Amphiphilic helices are a much discussed commonly occurring feature of both haemolytic peptides and mitochondrial presequences [13,5]. We think it likely that it is the interaction of the hydrophobic face of the helix with the membrane which differentiates the aggregating properties of p25 and melittin from that of agents such as polylysine.

Our experimental results further highlight the similarities between p25 and melittin. However, these similarities, as well as those previously reported by others [3], have been detected only in model systems. Further work involving mitochondrial membranes will be required to establish whether the melittin-like properties of p25 are relevant to protein translocation. Nevertheless, integral protein aggregation is likely to be a general phenomenon of its interaction with biological membranes. To our knowledge there is no reason why band 3 should be exceptionally disposed to such aggregation: separate studies have shown melittin at least, to be capable of aggregating bacteriorhodopsin in a reconstituted membrane system [19].

Opinion is currently divided on whether protein translocation can be understood simply in terms of interaction of the presequence and following peptide chain with the lipid bilayer, or whether a protein component of the membrane is required [3,20,21]. Previous models have been put forward which invoke the formation of a 'pore' by aggregation of integral membrane proteins by signal peptides [22,23]. The present studies demonstrate the feasibility of such a protein aggregation and suggest that further consideration should be given to this type of mechanism.

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