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Protein rotational diffusion measurements on the interaction of bee venom melittin with bacteriorhodopsin in lipid vesicles

Kun-Sheng Hu *, Mark J. Dufton **, Ian E.G. Morrison and Richard J. Cherry

Department of Chemistry, University of Essex, Wivenhoe Park, Cochester, Essex, CO4 3SQ (U.K.)

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The rotational diffusion of bacteriorhodopsin reconstituted into dimyristoylphosphatidylcholine vesicles was measured by the technique of flash-induced transient dichroism. In the presence of melittin, a cell lysing peptide from honey bee (*Apis mellifera*) venom, dose-dependent loss of rotational mobility was observed. Chemically modified melittin derivatives, in which free amine groups were either acetylated or succinylated, were impaired in their ability to induce immobilisation of bacteriorhodopsin. Bacteriorhodopsin reconstitutions of differing lipid/protein ratio were tested and it was found that the bacteriorhodopsin immmobilisation phenomena depended on the melittin/protein ratio, not the melittin/lipid ratio. This suggests that melittin produces its effect via direct interaction with bacteriorhodopsin. A mechanism is proposed in which the aggregation of bacteriorhodopsin is induced by electrostatic attraction between its anionic surface moieties and the highly cationic C-terminal segment of melittin.

Introduction

The venom of the honey bee (*Apis mellifera*) contains a potent toxin, termed melittin, which is able to lyse a variety of cell membranes at very low concentrations. It is a peptide of 26 amino acids, the residues bestowing on the molecule a pronounced amphiphilic character and a high degree of positive charge [1]. The secondary, tertiary and quaternary structures of melittin are variable, depending on concentration, counterions and solution dielectric constant [2–7]. In essence, however, melittin adopts an α-helical conformation, both in aqueous solution and at its site of action.

The relative structural simplicity of melittin, coupled with its dramatic effects on cell membranes, has prompted much investigation into its mode of action, which cannot be explained in simple surface-activity terms [1]. Various mechanisms have been proposed in which melittin-lipid interactions are considered foremost in the events leading to lysis [8–10], but the possibility that melittin-membrane protein interactions are involved has received little attention. We have anticipated that the profound effects of melittin on cell membranes might be sensed by the intrinsic proteins present since these are also integral components. Indeed, we have already shown via rotational diffusion measurements that melittin is able to immobilize band 3, a major protein component of the human erythrocyte membrane, and have offered an explanation as to the mechanistic significance [11].

In the present report, we continue this approach by investigating the effect of melittin on a simple model system consisting of bacteriorho-

* Permanent address: Biophysics Institute, Acadimia Sinica, Beijing, China.
** Present address: Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Building, Cathedral Street, Glasgow, U.K.

dopsin reconstituted into lipid vesicles. Rotational diffusion of bacteriorhodopsin may be measured by observing flash-induced transient dichroism of its intrinsic retinal chromophore [12–14]. The mobility and state of association of bacteriorhodopsin in dimyristoylphosphatidylcholine (DMPC) vesicles has previously been extensively investigated [12–19]. The purpose of the present study was to see whether the immobilising effect of melittin detected in the erythrocyte membrane is also present in this model system. Moreover, the ability to vary the lipid/protein mole ratio (L/P) in such a system enables us to test whether melittin-lipid or melittin-protein interactions are principally responsible for any observed effects.

Materials and Methods

(a) Preparation of bacteriorhodopsin / DMPC vesicles

The reconstitution method followed that described in detail by Cherry et al. [17]. Briefly, bacteriorhodopsin-containing purple membranes (12 mg) were isolated from *Halobacterium halobium* (strain R_1M_1) and solubilised through suspension in sodium acetate buffer (100 mM, pH 5.0, 48 ml) containing sodium azide (0.02%) and Triton X-100 (48 mg) for 24–30 h in the dark at room temperature. After centrifugation to remove unsolubilised residue, DMPC was added and the Triton removed by dialysis against sodium acetate buffer (100 mM, pH 5.0) containing sodium azide (0.02%) for about one week. The reconstituted sample was purified by sucrose density gradient centrifugation (15–40%) to remove remaining free lipid and protein. This method yields predominantly unilamellar lipid-protein vesicles with diameters in the range 0.3–0.5 μm. Protein content was determined by the method of Lowry et al. [20] and corrected for bacteriorhodopsin as described by Rehorek and Heyn [21]. Lipid content was determined by phosphorus analysis [22].

(b) Preparation of melittin derivatives

Native melittin was purified in the laboratory of Dr. R.C. Hider from whole *Apis mellifera* venom (Hider, R.C. and Dotimas, E., unpublished). Throughout the study, melittin concentration was determined according to a molar extinction coefficient of 5600 cm^{-1} at 280 nm. Succinyl and acetyl melittin derivatives in which the free amino groups are modified, were prepared by methods based on those of Habermann and Kowallek [23]. The indole absorption spectrum was virtually the same for native melittin and its two derivatives, so the same extinction coefficient was used in concentration determinations. Sample purity was analysed by HPLC as described elsewhere and there was no detectable phospholipase A_2 activity [11].

The incorporation of melittin and its derivatives in vesicles was monitored by centrifuging the vesicles and determining the melittin remaining in the supernatant.

(c) Measurement of bacteriorhodopsin rotation

The rotational diffusion of bacteriorhodopsin in the DMPC vesicles was measured by observing flash-induced transient dichroism. All measurements were made at 30°C, well above the temperature of the gel to liquid-crystalline phase transition (23°C) in DMPC. The transient dichroism apparatus used has been described previously [24]. Briefly the sample was excited by a vertically-polarised 532 nm laser pulse from a Nd-YAG laser (J K Lasers, Ltd.). Measurements of vertically and horizontally polarised transient intensity changes at 570 nm, due to ground state depletion, were obtained simultaneously with two photomultipliers. The signals were amplified, collected and averaged with a Datalab DL102A signal averager. Typically, 512 signals were collected in an individual experiment. The digitised decay curves were transferred to a Hewlett Packard 9825A desktop computer, where the intensity changes were converted to absorbance changes $A_{\parallel}(t)$ and $A_{\perp}(t)$ for polarisations parallel and perpendicular, respectively, to the exciting polarisation. The anisotropy parameter $r(t)$ was calculated as follows

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

For a homogeneous population of membrane proteins rotating only about an axis normal to the

plane of the membrane, $r(t)$ is given by

$$r(t) = \left[\frac{r_0}{A_1 + A_2 + A_3}\right] \times \left[A_1 \exp(-t/\phi_{\parallel}) + A_2 \exp(+4t/\phi_{\parallel}) + A_3\right] \quad (2)$$

where r_0 is the experimental value of the anisotropy at $t = 0$, $\phi_{\parallel}$ is the rotational relaxation time ($\phi_{\parallel} = 1/D_R$ where D_R is the rotational diffusion coefficient for rotation about the membrane normal) and A_1, A_2 and A_3 are constants which depend on the orientation of the transition dipole moment of the absorption band. It has been shown that Eqn. 2 is obeyed by bacteriorhodopsin in DMPC above the lipid phase transition provided the lipid/protein ratio (L/P) is sufficiently high to completely dissociate the bacteriorhodopsin into monomers [18]. However, at lower lipid/protein ratios, some association occurs resulting in multiple rotating species, each of which adds two further exponential terms to the anisotropy decay. In this case, $r(t)$ can at best be analysed by the general double-exponential equation

$$r(t) = r_1 \exp(-t/T_1) + r_2 \exp(-t/T_2) + r_3 \quad (3)$$

Because melittin apparently promotes association of bacteriorhodopsin, we found it appropriate to use Eqn. 3 throughout for data analysis. The experimental points were fitted by Eqn. 3 using a non-linear least-squares program on either a Hewlett Packard 9825A or BBC Model B desktop computer.

Results and data analysis

The absorption anisotropy decays of reconstituted bacteriorhodopsin at different concentrations of native melittin are shown in Fig. 1. Curve (i) is $r(t)$ obtained in the absence of melittin and shows an initial decay followed by a time-independent residual anisotropy [18]. As indicated under Methods, the curve is that expected when protein rotation occurs only about an axis perpendicular to the membrane. The curves obtained with increasing melittin concentration demonstrate successive retardations in the rate of the decay, thereby implying that decreases in the rotational motion of bacteriorhodopsin have occurred (curves (ii)–(vi), Fig. 1). The responses of bacteriorhodopsin to the acetyl and succinyl melittin derivatives are shown in Figs. 2 and 3, respectively. It transpires that both derivatives are impaired in their ability to immobilise bacteriorhodopsin, the succinyl melittin especially so. In the case of succinyl melittin, a concentration of $2.63 \cdot 10^{-5}$ M had little effect on bacteriorhodopsin rotation although

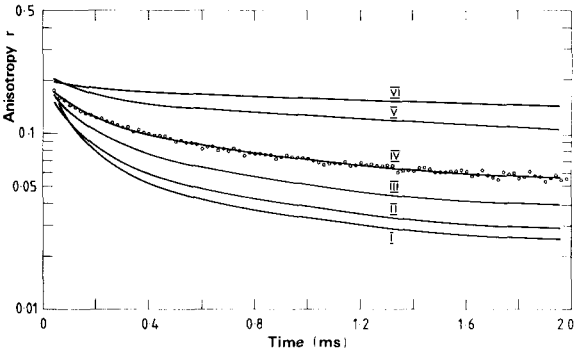

Fig. 1. Anisotropy decay curves for reconstituted bacteriorhodopsin in DMPC vesicles at different concentrations of native melittin. $r(t)$ was measured at 30°C with vesicles suspended in sodium acetate buffer (0.1 M, pH 5.0) containing sodium azide (0.02%). Bacteriorhodopsin concentration was $7.04 \cdot 10^{-6}$ M and the lipid/protein mole ratio was 38. Melittin concentrations as follows: (i) control, (ii) $2.2 \cdot 10^{-6}$ M, (iii) $4.4 \cdot 10^{-6}$ M, (iv) $8.8 \cdot 10^{-6}$ M, (v) $1.75 \cdot 10^{-5}$ M, (vi) $3.5 \cdot 10^{-5}$ M. The curves were obtained by fitting the experimental points using Eqn. 3. Typical signal to noise is illustrated in curve (iv).

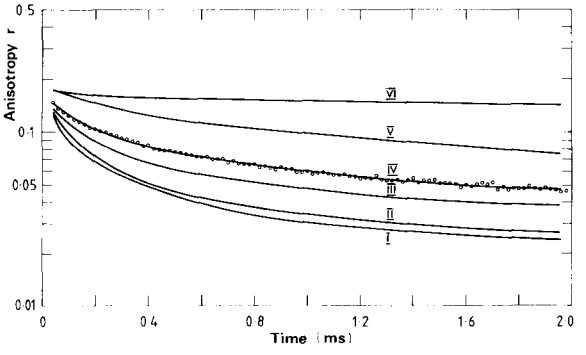

Fig. 2. Anisotropy decay curves for reconstituted bacteriorhodopsin at different concentrations of acetyl melittin. Experimental condition were as for Fig. 1. (i) control, (ii) $8.8 \cdot 10^{-6}$ M, (iii) $1.75 \cdot 10^{-5}$ M, (iv) $2.5 \cdot 10^{-5}$ M, (v) $3.5 \cdot 10^{-5}$ M, (vi) $5.35 \cdot 10^{-5}$ M.

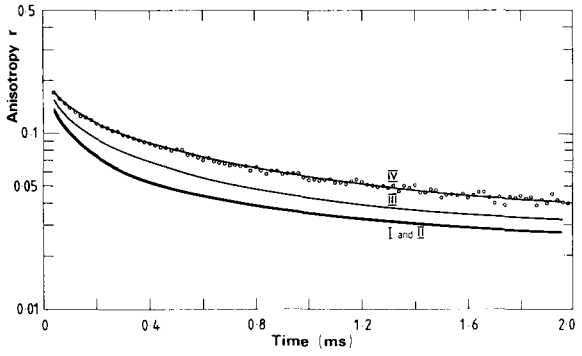

Fig. 3. Anisotropy decay curves for reconstituted bacteriorhodopsin at different concentrations of succinyl melittin. Experimental conditions were as for Fig. 1. (i) control, (ii) $2.63 \cdot 10^{-5}$ M, (iii) $3.5 \cdot 10^{-5}$ M, (iv) $5.35 \cdot 10^{-5}$ M.

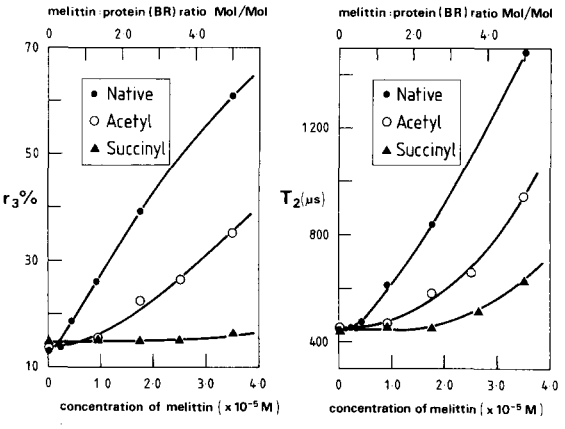

Fig. 4. The dependence of $r_3(\%)$ and T_2 on melittin concentration. The values of $r_3(\%)$ and T_2 were obtained by fitting the anisotropy decays in Figs. 1, 2 and 3 using Eqn. 3. ●, Native melittin; ○, acetyl melittin; △, succinyl melittin.

a comparable concentration of native melittin causes almost complete immobilization. A limiting factor in the measurements was the finding that at high concentrations, both native and acetyl melittin caused precipitation of the vesicles. For the samples used for the experiments shown in Figs. 1 and 2, it was not possible to abstract data for concentrations above $3.5 \cdot 10^{-5}$ M for native melittin and $5.4 \cdot 10^{-5}$ M for acetyl melittin.

When the anisotropy decays were fitted by Eqn. 3, no clear information was obtainable from the variation of parameters r_1, r_2 and T_1 with melittin concentration. The parameter T_2, however, did change smoothly. It should be noted here that when multiple rotating species are present, the resolution of $r(t)$ into two decay terms is arbitrary and T_1 and T_2 can only be regarded as approximate average relaxation times for these species. The most useful parameter is r_3 which is related to the fraction of bacteriorhodopsin which is immobile over the experimental time range of 2 ms. Fig. 4 shows plots of $r_3\%$ (i.e. 100 r_3/r_0 where r_0 is the anisotropy at $t = 0$) as a function of concentration for native, acetylated and succinylated melittin. The order of melittin immobilising efficacy is clearly shown to be native > acetyl > succinyl. Similarly, Fig. 4 also shows the variation of T_2 with melittin concentration, and as with $r_3\%$, the same rank order of efficacy is observed.

All the data in Figs. 1–4 were obtained using the same batch of reconstituted bacteriorhodopsin, in which the lipid/protein mole ratio was 38 and bacteriorhodopsin concentration was $0.7 \cdot 10^{-5}$ M.

Experiments similar to those shown in Fig. 1 were performed for three further samples of reconstituted vesicles of different lipid/protein ratio and a similar immobilising effect of melittin was observed. Table I summarises the effect of native melittin on $r_3\%$ in these samples. In Table I(a), the bacteriorhodopsin concentration was fixed at $0.5 \cdot 10^{-5}$ M, so that with varying lipid/protein ratio, the melittin/lipid ratio varied while the melittin/bacteriorhodopsin ratio remained constant. In contrast, in Table I(b), the lipid concentration was fixed at $7.75 \cdot 10^{-4}$ M so that the melittin : bacte-

TABLE I

IMMOBILIZATION OF BACTERIORHODOPSIN BY NATIVE MELITTIN AT DIFFERENT LIPID/PROTEIN RATIOS

(a) Experiments at constant melittin/protein ratio. (b) Experiments at constant melittin/lipid ratio. Note: L/P, Melittin/L and Melittin/P are mole ratios, $\Delta r_3\%$ is the change in $r_3\%$ from the control value. L, lipid; P, protein.

	L/P	Melittin/L	Melittin/P	$\Delta r_3\%$
(a)	50	0.063	3.2	23.5
	99	0.032	3.2	22.7
	161	0.020	3.2	21.4
(b)	50	0.021	1.0	8.6
	99	0.021	2.0	19.0
	161	0.021	3.3	24.2

riorhodopsin ratio varied while the melittin : lipid ratio remained constant.

When vesicles were centrifuged in the presence of the concentrations of melittin and its derivatives used in the rotation experiments, no melittin was detectable in the supernatant. Thus all the added melittin is associated with the vesicles.

Discussion

The central finding of this study is that bee venom melittin has a pronounced ability to decrease the rotational mobility of reconstituted bacteriorhodopsin. It should be noted that there is negligible contribution to the anisotropy decay by vesicle tumbling in DMPC vesicles [18]. This has recently been further confirmed by the observation that $r(t)$ does not significantly change when the aqueous phase viscosity is increased by glycerol or sucrose (Hu, K.-S., unpublished). Thus the changes in $r(t)$ induced by melittin cannot be assigned to reduced vesicle tumbling arising from fusion or aggregation of vesicles.

There would appear to be only two mechanisms whereby melittin could cause loss of rational mobility of bacteriorhodopsin in DMPC vesicles. One possibility is that melittin changes the physical state of the lipids which causes lipid-protein segregation and association of bacteriorhodopsin molecules. This is a plausible mechanism since rather similar effects have been observed upon either incorporating cholesterol [16] into the bilayer or lowering the temperature [17]. It should be noted that melittin does not change the transition temperature of DMPC although it does increase the lipid order in the fluid phase [25]. The other possibility is that melittin binds directly to bacteriorhodopsin and effectively crosslinks the protein into larger aggregates. Both mechanisms, it should be noted, assume that the loss of mobility is due to association of bacteriorhodopsin, since it is highly improbable that the large effects observed could be accounted for by changes in membrane viscosity alone.

The experiments with vesicles of different lipid/protein ratios were performed to permit a distinction between the above two mechanisms. The results in Table I show clearly that the degree of bacteriorhodopsin immobilization is determined by the melittin/protein ratio rather than the melittin/lipid ratio. This argues strongly for a direct interaction between melittin and bacteriorhodopsin. The nature of this proposed interaction, however, depends on the interpretation placed upon the reduced abilities of acetyl melittin and succinyl melittin to produce the immobilisation effect.

The major difference between native melittin and its two derivatives is overall charge at the experimental pH. Whereas native melittin possesses six basic moieties (two arginines, three lysines and the N-terminus), acetyl melittin has a residual charge of 2 + (two arginines) and succinyl melittin of 2 − (two arginines, three succinylated lysines and one succinylated N-terminus). In a peptide of 26 residues, these changes will have significant consequences for conformation as well as function. Indeed, it has been shown via circular dichroism that these modifications induce greater proportions of α-helix in the molecule [26]. Although there is evidence to show that native melittin adopts a predominantly α-helical structure in the membrane [4,5,7,27], α-helix formation in free solution can also indicate the formation of melittin tetramer [27]. Native melittin is not active in its tetrameric form [27], principally because the hydrophobic areas of the melittin monomer are shielded from the lipid phase by virtue of the quaternary structure. In accounting for the reduced efficacy of acetyl melittin and succinyl melittin, therefore, an increased degree of tetramer stability could reduce accumulation in the bilayer.

To answer this question, the ability of succinyl melittin, the least active derivative, to partition between the aqueous phase and the bacteriorhodopsin vesicles was measured against that of native melittin. No significant difference was noted, so it appears that while both native and succinyl melittin can accumulate in the membrane, the interaction of the latter with bacteriorhodopsin has become greatly reduced.

Bacteriorhodopsin and melittin possess both hydrophobic segments and charged hydrophilic moieties that reside in the aqueous phase. In the case of bacteriorhodopsin, the molecule protrudes from both sides of the bilayer, one side bearing an overall charge of 1 − and the other side of 7 − [28]. In the absence of melittin, bacteriorhodopsin

could be dispersed in the vesicle membrane by a degree of anionic repulsion between these charged regions. (A similar explanation has been proposed for glycophorin dispersion in the erythrocyte membrane [29]). The major hydrophilic segment of melittin is the C-terminal segment of six residues that bears a cluster of four positive charges. A plausible mechanism of bacteriorhodopsin aggregation is thus one in which melittin is embedded in the membrane via its hydrophobic segment while the cationic C-terminus crosslinks bacteriorhodopsin by electrostatic binding to anion residues. Some degree of hydrophobic interaction between the intramembrane portions of bacteriorhodopsin and melittin is also a possibility, but this would probably be secondary to the ionic aspect.

Previously, we have shown that melittin immobilizes band 3, a major protein component of the human erythrocyte membrane [11]. Acetylation and succinylation of melittin change its ability to immobilize band 3 in a similar manner to that reported for bacteriorhodopsin in the present study. These similarities give good reason to suppose that essentially the same aggregation mechanism as outlined above may be involved also in the case of band 3.

Taken together, the reconstituted bacteriorhodopsin and band 3 studies have revealed a new and significant facet of melittin action on membranes. Despite the differences between bacteriorhodopsin and band 3, both can be readily immobilised by melittin. Thus the protein immobilizing property of the toxin is relatively nonspecific. If our proposed mechanism is correct, the only requirement is for the protein to have negative charges close to the membrane surface. This may well be a rather general property of membrane proteins. Melittin is active against a variety of cell types [1], so a 'universal' mechanism of the kind proposed would seem to be quite appropriate.

The significance of the aggregation effects for the mode of action of melittin on biological membranes is two-fold. Firstly, where direct lysis of cells by melittin alone is involved, the mechanism may depend on melittin-intrinsic protein interactions as well as melittin-lipid interactions. Secondly, they provide a basis for a fuller understanding of the high degree of synergism that exists between melittin and phospholipase A_2, the second major component of bee venom, in cell lysis. Whilst synergism between melittin and phospholipase A_2 undoubtedly occurs in pure lipid systems [30–32], there are significant differences in behaviour between these systems and cell membranes. Thus succinylation of melittin totally abolishes synergism in erythrocyte lysis [11] but only reduces the effect by 50% in lipid vesicles [32]. In cells, uniformly distributed intrinsic membrane proteins may restrict the access of enzymes like phospholipase A_2 to the lipids, especially as many of these proteins have large extracellular carbohydrate moieties attached. The ability of melittin to aggregate these proteins will disrupt this uniformity and will probably increase the surface area of lipid available to the enzyme, thereby permitting the latter to realise its full potential as a lytic agent [11].

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