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Calcium-regulated interactions of human α-lactalbumin with bee venom melittin

Eugene A. Permyakov [a], Valery M. Grishchenko [a], Lina P. Kalinichenko [a], Nikolay Y. Orlov [a], Kunihiro Kuwajima [b] and Shintaro Sugai [b]

[a] *Institute of Biological Physics, U.S.S.R. Academy of Sciences, Pushchino, Moscow Region 142292, U.S.S.R.* and [b] *Department of Polymer Science, Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060, Japan*

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Affinity chromatography, fluorescence and circular dichroism spectroscopy methods have been used to study the interaction of melittin, a 26-residue peptide from bee venom, with Ca^{2+}-binding α-lactalbumin from human milk. It has been revealed that melittin binds to the apo- and acidic states of α-lactalbumin while the presence of Ca^{2+} makes the interaction essentially weaker. The association constant for the complex of melittin with apo-α-lactalbumin determined from spectropolarimetric melittin-titration data is 2×10^7 M^{-1}. The complexation of α-lactalbumin with melittin decreases its affinity to Ca^{2+} by three orders of magnitude. The interaction of apo-α-lactalbumin with melittin causes some changes in the environment of its aromatic amino acid residues and drastically alters the conformation of melittin, increasing its α-helical content but leaving its single tryptophan residue accessible to water. In the case of the acidic state of α-lactalbumin the interaction does not induce an increase in α-helical content of melittin.

1. Introduction

It is well known now that some calcium-binding proteins interact with short peptides. As a rule, such interactions are Ca^{2+}-dependent, i.e., they take place only in the presence of Ca^{2+}. For example, calmodulin, troponin C and myosin light chains display Ca^{2+}-dependent interactions with peptides exhibiting common structural features, notably clusters of basic amino acid residues in close conjunction with hydrophobic sequences [1–5]. One such peptide is melittin, a 26-residue peptide from honey bee venom. Melittin interacts with Ca^{2+}-loaded forms of calmodulin and troponin C with very high affinity (dissociation constant about 0.1 nM) to yield a 1 : 1 complex in which melittin exists in an α-helical conformation [1,2]. The interactions results in a shift of the emission band of Trp-19 of melittin towards shorter wavelengths.

Recently, we have revealed that melittin also interacts with parvalbumin, however, the interaction takes place only in the absence of divalent cations [6]. The binding of melittin to apo-parvalbumin also results in a shift to longer wavelengths of the emission band of melittin. In the present work, we have found that there is another Ca^{2+}-binding protein, human α-lactalbumin, which interacts with melittin also only in the absence of Ca^{2+}. Although such peptide-protein associations probably do not occur in vivo, they proved to be useful models for protein-protein interactions.

Correspondence address: E.A. Permyakov, Institute of Biological Physics, U.S.S.R. Academy of Sciences, Pushchino, Moscow Region 142292, U.S.S.R.

2. Materials and methods

α-Lactalbumin was isolated and purified from human milk as described by Kaplanas and Antanavichius [7]. Melittin was isolated and purified from bee venom by the method described by Te Plao King et al. [8]. The purity of the protein preparations was checked electrophoretically.

Melittin-agarose was prepared according to the method of Kincaid and Coulson [9]. Affinity chromatography on melittin-agarose was carried out in a plastic column containing 5 ml melittin-agarose (0.6 mg melittin per ml gel) at a flow rate of 28 ml per h. 1.34 mg of α-lactalbumin dissolved in 3 ml of the corresponding buffer was applied to the column. 2.5-ml fractions were collected at 5-min intervals. Ultraviolet absorption of the fractions was monitored by means of a Uvicord S instrument. Steady-state fluorescence measurements were carried out on a laboratory-built spectrofluorimeter described earlier [10]. Fluorescence light was collected from the front surface of the quartz cell. All spectra were corrected for instrumental spectral sensitivity of the spectrofluorimeter. Intensities in the corrected spectra were proportional to the number of photons emitted per unit wavelength interval.

CD measurements were carried out with a Jasco Model 500A spectropolarimeter. The spectra were recorded at protein concentrations of 0.1–0.7 mg/ml using cells with path lengths of 0.1, 1.0 and 10.0 cm.

Ultraviolet absorption spectra were measured with a Specord UV-Vis (Karl Zeiss, Jena) or Shimadzu UV-3000 spectrophotometer.

Fitting of the experimental data with theoretical curves was carried out with a computer using a non-linear regression scheme [11]. The accuracy in evaluation of the binding constants by the fitting procedure was about half an order of magnitude.

3. Results and discussion

It is well known that the α-lactalbumin molecule possesses one high-affinity Ca^{2+}-binding site [12,13] and several separate Zn^{2+}-binding sites [14,15]. Release of the bound Ca^{2+} from the protein makes its structure looser and less compact [16]. The binding of Zn^{2+} or Cu^{2+} to Ca^{2+}-loaded α-lactalbumin produces a similar effect [15,17]. At low pH values, α-lactalbumin undergoes a so-called acidic conformational change and its acidic state in some respects is similar to the apo state [13,16].

In the present work, we have studied the possibility of interactions of melittin with various states of α-lactalbumin. Fig. 1 and table 1 show the results from chromatography of α-lactalbumin in different states on the melittin-agarose column. It is clearly seen from the data obtained that Ca^{2+}-loaded and Zn^{2+},Ca^{2+}-loaded α-lactalbumin practically do not interact with immobilized melittin. In contrast, the acidic state of α-lactalbumin and especially the apo state of α-lactalbumin bind to immobilized melittin very tightly. The same results were obtained in the presence of 150 mM NaCl and 5 mM $MgCl_2$ at pH 7.2. Washing of the

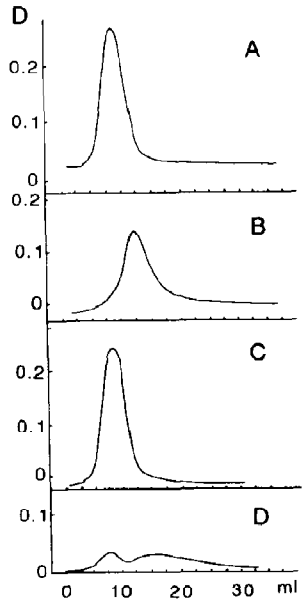

Fig. 1. Chromatography of α-lactalbumin in different states on melittin-agarose: (A) Zn^{2+},Ca^{2+}-state (5 mM $CaCl_2$, 6.6×10^{-5} M $ZnCl_2$); (B) acidic state (25 mM glycine, pH 2.59); (C) Ca^{2+}-loaded state (1 mM $CaCl_2$); (D) apo state (4 mM EGTA). 25 mM Mops (pH 8.2), 1 mM azide used in all experiments except (B); 20°C. Initial protein concentration, 3.3×10^{-5} M.

Table 1

Proportion of human α-lactalbumin adsorbed on melittin-agarose column in different conditions

Initial concentration of the protein was 3.3×10^{-5} M

State of α-lactalbumin	Adsorbed α-lactalbumin (%)
Ca^{2+} state (25 mM Mops, pH 8.2; 1 mM $CaCl_2$)	11
Apo state (25 mM Mops, pH 8.2; 4 mM EGTA)	90
Zn^{2+},Ca^{2+} state (25 mM Mops, pH 8.2; 5 mM $CaCl_2$, 6.6×10^{-5} M $ZnCl_2$)	1
Acidic state (25 mM glycine, pH 2.57)	53

column with bound apo-α-lactalbumin by Ca^{2+}-containing buffer or 6 M urea does not release all the bound protein. Complete removal of the bound protein requires several repetitions of the washing procedure.

As mentioned above, the binding of melittin to calmodulin, troponin C and parvalbumin results in a shift of the tryptophan fluorescence spectrum of melittin towards shorter wavelengths. We have attempted to demonstrate this effect in the case of the interaction of melittin with apo-α-lactalbumin. The main difficulty in this case is that both proteins have tryptophan residues. We performed spectrofluorometric titration of melittin by apo-α-lactalbumin. The fluorescence spectra of mixtures of melittin and α-lactalbumin were fitted by the sum of the spectra of apo-α-lactalbumin and free and bound melittin. The spectrum of the protein-bound melittin was that of melittin bound to pike parvalbumin which is devoid of tryptophan residues [6]. It transpired that all the spectra were best fitted by only two components, corresponding to the emission spectra of apo-α-lactalbumin and free melittin. Fig. 2 shows the quality of the fit for a 1 : 1 mixture of the proteins. Fig. 3 demonstrates the dependence of the contributions of the spectra of apo-α-lactalbumin and free and bound melittin to the total fluorescence spectrum on the relative concentration of α-lactalbumin. The results obtained show that the interaction of melittin with apo-α-lactalbumin does not induce a blue shift of the melittin fluorescence spectrum,

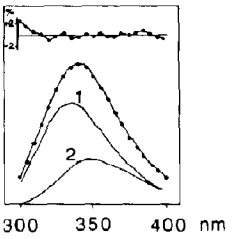

Fig. 2. Fitting of the fluorescence spectrum of a 1 : 1 mixture of melittin with apo-α-lactalbumin by emission spectra of apo-α-lactalbumin (1) and free melittin (2). In the upper part of the figure the differences between the experimental and theoretical values are shown. 25 mM Hepes, (pH 8), 20 °C.

i.e., the transfer of its single tryptophan residue to a more hydrophobic environment does not take place in this case. This means that the mechanism of interaction of melittin with α-lactalbumin is somewhat different from that which takes place in the case of the interaction with calmodulin, troponin C and parvalbumin. Furthermore, no spectral shift of the melittin fluorescence spectrum was observed for the Ca^{2+}-loaded, Zn^{2+},Ca^{2+}-loaded and acidic states of α-lactalbumin.

As mentioned above, melittin in the complexes with calmodulin and troponin C exists in the α-helical conformation. We attempted to establish whether this also occurs for the complex of melittin with α-lactalbumin. Fig. 4 shows CD spectra in the peptide and aromatic regions of melittin, α-lactalbumin and their mixture in the presence of

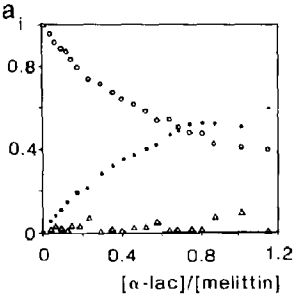

Fig. 3. Dependence of contributions of emission spectra of α-lactalbumin (●) and free (○) and bound (△) melittin to the total fluorescence spectrum on relative concentration of apo-α-lactalbumin. 25 mM Hepes (pH 8), 20 °C. $a_i = S_i / \Sigma S_i$, where S_i is the area under the emission spectrum of component i.

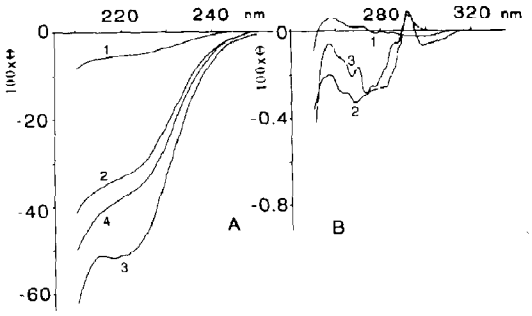

Fig. 4. CD spectra in the far- (A) and near- (B) ultraviolet regions of 5.7×10^{-5} M melittin (1), 2.7×10^{-5} M α-lactalbumin (2) and their mixture (3) in 25 mM Tris-HCl (pH 8), 3.2 mM EGTA at 20 °C. Curve 4 is the sum of curves 1 and 2. θ, observed ellipticity in degree/cm; molar ellipticity, calculated from 100×[observed ellipticity]/[molar concentration of peptide or protein].

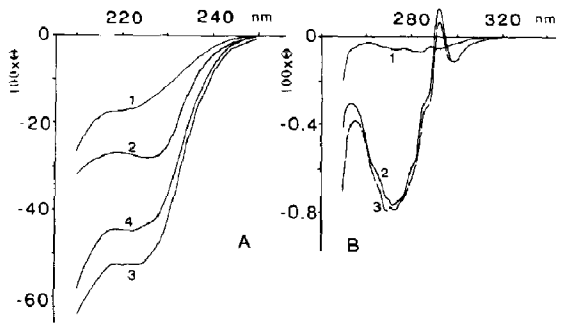

Fig. 5. CD spectra in the far- (A) and near- (B) ultraviolet regions of 1.7×10^{-4} M melittin (1), 2.7×10^{-5} M α-lactalbumin (2) and their mixture (3) in 25 mM Tris-HCl (pH 8), 3.2 mM $CaCl_2$ at 20 °C. Curve 4 is the sum of curves 1 and 2. θ, observed ellipticity (degree/cm).

3.2 nM EGTA. It is clearly seen from fig. 4A that the interaction of apo-α-lactalbumin with melittin results in a considerable increase in ellipticity in the peptide region which amounts to more than the sum of the curves for free melittin and apo-α-lactalbumin. Such an increase in ellipticity appears to be due to the rise in α-helical content of bound melittin. Fig. 4B shows that the interaction alters the environment of some phenylalanine and tyrosine chromophores in apo-α-lactalbumin and perhaps in melittin, however, it does not modify that of the tryptophan residues, being consistent with the results for the tryptophan fluorescence spectra.

Therefore, the results obtained by fluorescence and CD methods demonstrate that the interaction of apo-α-lactalbumin with melittin induces a number of changes in the structure of α-lactalbumin and drastically changes the conformation of melittin, increasing its α-helical content but leaving its single tryptophan residue accessible to water.

Fig. 5 shows CD spectra of melittin, α-lactalbumin and their mixture in the presence of 3.2 mM $CaCl_2$. It is evident that the spectrum of the mixture is not the sum of the spectra of free melittin and α-lactalbumin, however, the difference between them is not as pronounced as in the case of apo-α-lactalbumin in spite of the fact that in this experiment the melittin/α-lactalbumin molar ratio was 6.2 and with the apo-protein 2.1. This appears to reflect the lower affinity of the Ca^{2+}-loaded α-lactalbumin to melittin in comparison with the apo-protein. Fig. 5B shows that the changes in the environment of the aromatic amino acid residues in Ca^{2+}-loaded α-lactalbumin are very slight in the case of its interactions with melittin.

CD spectra of melittin, α-lactalbumin and their mixture at pH 2.55 are shown in fig. 6. In this case the interaction even decreases the ellipticity in the peptide region in comparison with the sum of the two curves for free melittin and α-lactalbumin. Nevertheless, the interaction does induce small

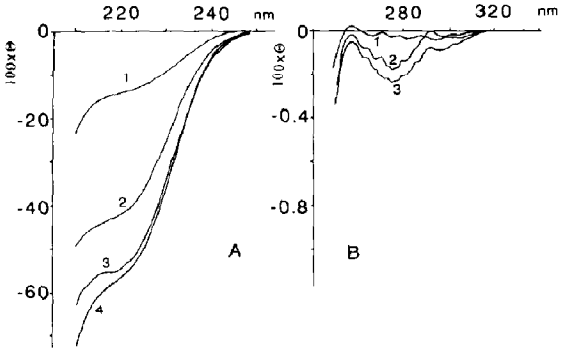

Fig. 6. CD spectra in the far- (A) and near- (B) ultraviolet regions of 1.6×10^{-4} M melittin (1), 2.5×10^{-5} M α-lactalbumin and their mixture (3) at pH 2.55 and 20 °C. Curve 4 is the sum of curves 1 and 2. θ, observed ellipticity (degree/cm).

changes in the environment of the aromatic amino acid residues of α-lactalbumin. Hence, the mechanism of interaction of melittin with the acidic state of α-lactalbumin seems to differ from that for apo- and Ca^{2+}-loaded α-lactalbumin at neutral pH values: perhaps in this case melittin in the complex with the protein does not exist in the α-helical conformation.

In order to evaluate quantitatively the parameters of the interaction of apo-α-lactalbumin with melittin, we carried out spectrofluorimetric titration of α-lactalbumin by melittin. The results of these experiments are illustrated in figs 7 and 8. These figures show the dependence of the fluorescence intensity at a fixed wavelength on the $(1 - T)$ value ($T = 10^{-D}$, D being the absorbance at the excitation wavelength, 280.4 nm). Both melittin and α-lactalbumin contain tryptophan residues and if the proteins do not interact with each other, the fluorescence intensity of their mixture will be proportional to $(1 - T)$ (the fluorescence in our spectrofluorimeter is collected from the front surface of the cell). It is clearly seen from figs 7A and 8B that for Ca^{2+}-loaded and Zn^{2+},Ca^{2+}-loaded α-lactalbumin, the dependence of fluorescence intensity on $(1 - T)$ is linear in behaviour while in the case of apo-α-lactalbumin and its acidic state it deviates from linearity at low values of $(1 - T)$. This deviation from linearity indicates that at these concentrations melittin interacts with α-lactalbumin in solution and that the interaction modifies their fluorescence.

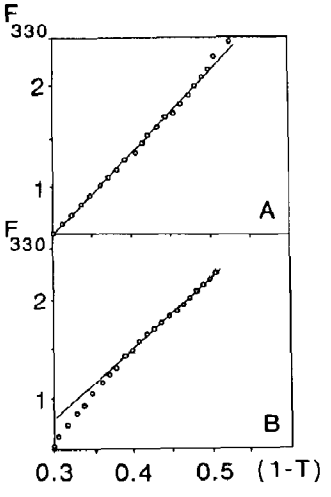

Fig. 7. Spectrofluorometric titration of α-lactalbumin by melittin. The data are represented in coordinates of fluorescence intensity at 300 nm vs $(1-T)$ ($T=10^{-D}$; D being the absorbance at the excitation wavelength of 280.4 nm). (A) Ca^{2+}-loaded α-lactalbumin (1 mM $CaCl_2$); (B) apo-α-lactalbumin (3 mM EGTA). 25 mM Hepes (pH 8.2), 20 °C.

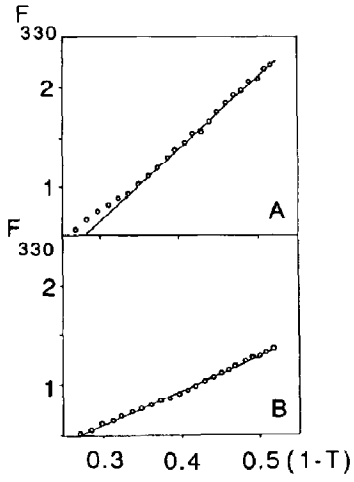

Fig. 8. Spectrofluorometric titration of α-lactalbumin by melittin. The data are represented in coordinates of fluorescence intensity at 335 nm vs $(1 - T)$. (A) Acidic state of α-lactalbumin (25 mM glycine, pH 2.59); (B) Zn^{2+},Ca^{2+}-α-lactalbumin (5 mM $CaCl_2$, 6.6×10^{-5} M $ZnCl_2$, 25 mM Hepes, pH 8.2) 20 °C.

Fig. 9 shows the dependence of the difference between the extrapolated linear parts of the fluorescence intensity vs $(1 - T)$ plots and experimental fluorescence intensity values on the relative concentration of melittin. The experimental data were fitted by a theoretical curve computed according to the simplest one-site binding scheme. The best fit was achieved when the association constant for the melittin-apo-α-lactalbumin complex was equal to 7×10^5 M^{-1}. In the case of the acidic state of α-lactalbumin, the best fit is achieved when the association constant is 8×10^5 M^{-1}. Since the fluorescence effect in these experiments was rather small, the accuracy of these evaluations was low. Therefore, we tried to determine the association constant for the melittin-α-lactalbumin complex from the spectropolarimetric data. Fig. 10 depicts the rise in the observed

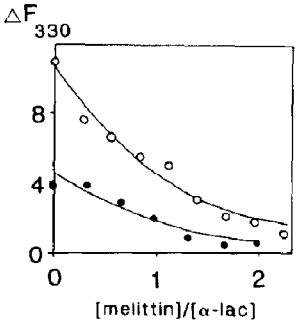

Fig. 9. Dependence of the difference between the extrapolated linear portion of the intensity vs $(1-T)$ plots and experimental fluorescence intensities on relative melittin concentration for apo-lactalbumin (○) and the acidic form of α-lactalbumin (○) obtained from figs 7B and 8A. The curve is the theoretical one computed according to the one-site binding scheme and fitted to the experimental points by variation of the association constant.

ellipticity of apo-α-lactalbumin at 200 nm with increase in the relative concentration of melittin. A control experiment on titration of a buffer solution by melittin showed that the increase in ellipticity due to the rise in concentration of free melittin is negligibly small in this case. Evaluation of the binding constant from these data led to a value of 2×10^7 M^{-1}. Since the spectropolarimetric effect is sufficiently large, the accuracy of this value is greater than that in the fluorometric measurements.

If a calcium-binding protein interacts with a compound, e.g., a peptide, and the interaction is modulated by Ca^{2+} binding, then the binding of Ca^{2+} in turn should be modulated by the binding of the compound. This conclusion can be drawn from a consideration of thermodynamics of the simple equilibrium binding scheme:

$$\begin{array}{ccc} P + M & \overset{K_m}{\rightleftharpoons} & P \cdot M \\ {\scriptstyle +Ca^{2+}}\uparrow\downarrow{\scriptstyle K_c} & & {\scriptstyle K_c^*}\uparrow\downarrow{\scriptstyle +Ca^{2+}} \\ Ca \cdot P + M & \underset{K_m^*}{\rightleftharpoons} & Ca \cdot P \cdot M \end{array} \qquad (1)$$

where P represents α-lactalbumin, M melittin, K_m and K_m^* denote the melittin binding constants for apo- and Ca^{2+}-loaded α-lactalbumin, respectively, and K_c and K_c^* correspond to the Ca^{2+}-binding constants for melittin-free and melittin-loaded α-lactalbumin. It is readily observed that

$$K_c/K_c^* = K_m/K_m^*$$

This signifies that if apo-α-lactalbumin has a higher affinity to melittin in comparison with the Ca^{2+}-loaded protein, then it must possess a higher affinity to Ca^{2+} in the absence of melittin than in its presence. We have examined this conclusion.

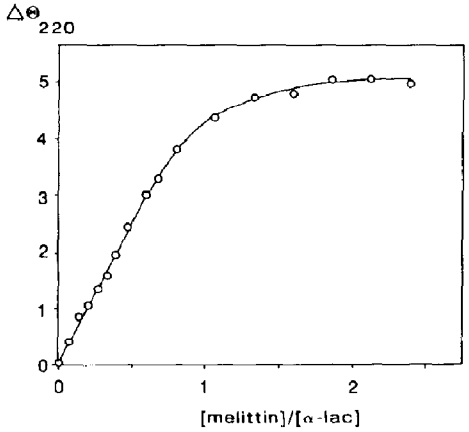

Fig. 10. Spectropolarimetric titration of 2.6×10^{-6} M apo-α-lactalbumin by melittin. 12.5 mM Tris-HCl (pH 8), 0.23 mM EGTA; 20 °C.

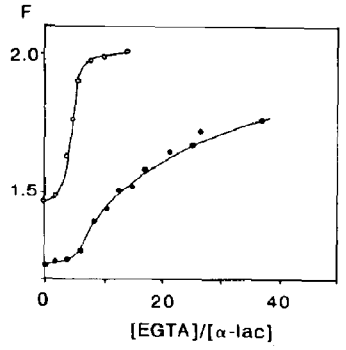

Fig. 11. Spectrofluorometric EGTA-titration of 9.4×10^{-6} M Ca^{2+}-loaded (12×10^{-6} M $CaCl_2$) human α-lactalbumin in the absence (●) and presence (○) of 1.44×10^{-5} M melittin. 25 mM Hepes (pH 8.2); 20 °C. The curves are theoretical ones computed according to the scheme, eq. 3, and fitted to the experimental points by variation of the Ca^{2+} association constant. F, relative fluorescence intensity at 350 nm.

Fig. 11 shows the results of the spectrofluorometric EGTA titration of Ca^{2+}-loaded α-lactalbumin in the presence and absence of melittin. It is clearly observed that the presence of melittin decreases the affinity of α-lactalbumin to Ca^{2+}. The experimental points in fig. 11 were fitted by theoretical curves computed according to the simple scheme of competition of the protein and EGTA for Ca^{2+}:

where P is protein, are K and K_e denote the Ca^{2+}-binding constants of the protein and EGTA, respectively. The fitting was carried out by variation of K. The best fit was obtained when $K = 1.2 \times 10^9\ M^{-1}$ for α-lactalbumin in the absence of melittin and $K = 2.3 \times 10^6\ M^{-1}$ for α-lactalbumin in the complex with melittin, i.e., the complexation of α-lactalbumin with melittin decreases its affinity to Ca^{2+} by three orders of magnitude. Taking into account the relationship, eq. 2, one can conclude that the melittin-binding constant for Ca^{2+}-loaded α-lactalbumin is $4 \times 10^4\ M^{-1}$.

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