gives eq 4, $pK_d = pK_d^0 - z_m^2\Phi$, where

$$\Phi = \frac{4e^2}{4.606kTD}\left[4\left(\frac{1}{b_T} - \frac{\kappa}{1 + \kappa a_T}\right) - \left(\frac{1}{b_M} - \frac{\kappa}{1 + \kappa a_M}\right)\right] \quad (23)$$

Registry No. Melittin, 20449-79-0.

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Conformational Studies of Aqueous Melittin: Determination of Ionization Constants of Lysine-21 and Lysine-23 by Reactivity toward 2,4,6-Trinitrobenzenesulfonate†

Steven C. Quay* and Lynn P. Tronson

ABSTRACT: The reaction of monomeric and tetrameric melittin with 2,4,6-trinitrobenzenesulfonate (TNBS) was studied as a function of pH to determine ionization constants for Lys-21 and -23 in melittin. The reaction is a simple second-order process and is well described by the rate equation, $d[P]/dt = k_{obsd}[\text{melittin}]_0[\text{TNBS}]_0$. Monomeric melittin at $\Gamma/2$ 0.01, 23 °C, contains two ionization constants with values of $10^{-6.5}$ M and and $10^{-8.6}$ M. The intrinsic reactivity of these groups differs by 6-fold, probably reflecting a difference in accessibility to the bulky TNBS. In tetrameric melittin at $\Gamma/2$ 2.53, 23 °C, the data support the assignment of two K_H values with a sum of $2 \times 10^{-7.4}$ M. The location of TNBS substitution was determined by cleavage of TNBS-melittin adducts with trypsin (at Arg and unsubstituted Lys residues) or with *o*-iodosobenzoic acid (at the single Trp residue) and by purification by Sephadex LH-20 column chromatography, acid hydrolysis, and thin-layer chromatography to identify free amino acids. The results indicate that the lysine residue with the pK of 6.5 is Lys-21 while Lys-23 has a pK of 8.6 in monomeric melittin. The failure to obtain substitution at Lys-7 at the highest pH values of this study indicates this residue has an ionization constant $\geq 10^{-9.6}$ M. In addition, the α-amino of glycine reacts with TNBS over 20 times more slowly than the lysines of melittin, precluding determination of its ionization constant by this technique.

The pharmacological and biochemical actions of the neurotoxin melittin have been extensively explored [see review in Habermann (1972)]. Studies of the action of melittin fragments indicate that the C-terminal sequence, -Lys_{21}-Arg-Lys-Arg-, is essential both for binding to and for interaction with lipid membranes (Verma et al., 1976; Dawson et al., 1978).

In this paper, we determine the ionization constants of the ϵ-amino groups of lysine residues 21 and 23. The clustered basicity of these residues led us to anticipate that the K_H values for these groups would be larger than those for typical ϵ-amino groups. In fact, the K_H values, measured by reactivity with 2,4,6-trinitrobenzenesulfonate (TNBS),[1] were as much as

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10 000 times larger than for normal lysine residues. The finding of pK values for lysine residues 21 and 23 between 6.5 and 8.6 for melittin in monomeric or tetrameric form suggests that changes in the state of ionization of these residues could play an important role in melittin–melittin and melittin–phospholipid interactions.

Experimental Procedures

Materials. 2,4,6-Trinitrobenzenesulfonic acid (tetrahydrate form) was obtained from Aldrich and used without further purification. Melittin, from *Apis mellifera*, was obtained from Sigma Chemical Co., St. Louis, MO. Melittin was used without purification or was purified as described in detail elsewhere (S. C. Quay and C. C. Condie, unpublished results). There was no significant difference in the data obtained with either preparation. *o*-Iodosobenzoic acid (Pierce Chemical Co., Rockford, IL) was stored desiccated at −20 °C. Trypsin (TPCK treated to inactivate chymotrypsin activity) was obtained from Millipore Corp., Freehold, NJ, and stored desiccated at 4 °C.

Methods. Spectra were recorded on a Hitachi 110 spectrophotometer at room temperature. Matched 1-cm quartz cuvettes were used for spectral and kinetic recordings. Spectra were corrected for buffer absorbance and cuvette irregularities by electronic auto base line. The kinetic records of the reaction of TNBS with melittin to form TNP-melittin bisulfite complexes were typically made in the following manner. To a clean, dry cuvette was added 1.00 mL of buffer, followed by 5–10 μL of a ca. 0.2 M TNBS solution at the same pH as the buffer. The small absorbance at 367 nm was electronically subtracted, and then 5–20 μL of a ca. 10^{-4} M melittin solution was rapidly added, the solution mixed thoroughly with a clean Pasteur pipette, and the chart recording begun at 0.3–5 min/1-in. division (0.2A full scale). The particular choice of recorder speed was set to optimize the precision in measuring both ΔA and Δtime. The mixing period was typically 3–6 s and always represented <15% of the total absorbance change. The reaction rate was determined at several $[\text{melittin}]_0$, typically over a 5-fold concentration range. The pH remained constant during the course of the reaction. The linear portion of the recording was used to estimate the concentration of TNBS adduct, 10 550 cm^{-1} M^{-1} being used as the molar absorptivity of ϵ-amino TNBS adducts (Means et al., 1972). The calculated stoichiometry of 3.6 ± 0.2 (n = 12) for TNBS–melittin at t_∞ indicated this extinction value is fairly accurate, since there are three lysine groups and an α-amino of glycine in melittin.

The structural elucidation of TNBS–melittin adducts was performed as shown schematically in Figure 1. TNBS–melittin was prepared as described above (but in larger quantities, typically 50–100 nmol) and separated from the reaction solution by Sephadex G-25 column chromatography in 0.05 M sodium phosphate, pH 7.15. The product was dried under a N_2 stream at 50 °C or by lyophilization.

Samples were reacted with o-iodosobenzoic acid (IOBA) at 23 °C for 18–24 h (1.0–2.5 mg of IOBA; 25–60 nmol of melittin or TNBS–melittin; 80% acetic acid; 4 M guanidine hydrochloride; 100–250 μL) to afford cleavage at the carboxy terminus of Trp-19, presumably by a two-step mechanism involving oxidation of the indole ring to an iminospirolactone and hydrolysis to yield an *N*-acyldioxindolylalaninyl-terminated peptide (MaHoney et al., 1981). The peptides were separated on a 1 × 50 cm Sephadex LH-20 column [ethanol–formic acid (3:1 v/v)] at room temperature.

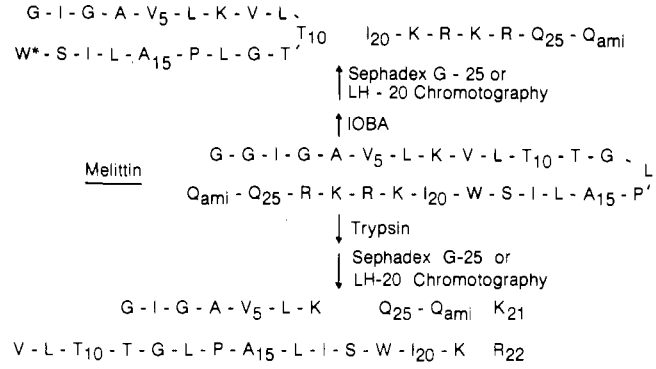


FIGURE 1: Schematic for structural determination of TNP derivatives of melittin. The fragments shown are those expected from trypsin or IOBA treatment of unsubstituted melittin. TNP substitution of lysine residues prevents trypsin cleavage.

Trypsin hydrolysis was performed as described (Smyth, 1967) in 0.02 M Tris-HCl, pH 8.0, room temperature (10 μg of trypsin; 25–60 nmol of melittin or TNBS–melittin; 100–250 μL) for 12–15 h. The peptides were separated initially by Sephadex LH-20 column chromatography as described above.

Thin-layer chromatography was performed (Niederwieser, 1972) on silica gel plates (250 μm) in solvent A [1-butanol–acetic acid–pyridene–water (15:3:9:12 v/v)] or solvent B [chloroform–methanol–17% ammonia (2:2:1 v/v)]. Peptides were identified as TNBS adducts by the visible yellow color and by quenching of the silica-bound zinc silicate phosphor when viewed with short-UV light, as tryptophan-containing peptides by fluorescence observed with a short-wavelength lamp, or as free, amino-containing fragments by dipping or spraying the slides in a ninhydrin solution (500 mg of ninhydrin–5 mL of acetic acid–100 mL of 1-butanol) and heating to 90 °C for several minutes.

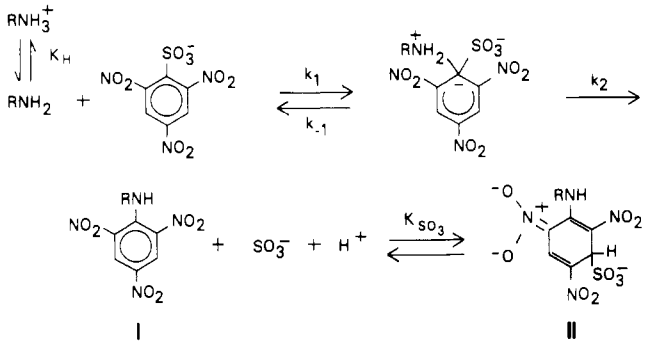
The identified fragments were scrapped from unstained silica gel slides run in parallel with ninhydrin-treated slides and eluted with water, dried under a N_2 stream, and acid hydrolyzed with 100 μL of HCl–propionic acid (50:50 v/v) at 160 °C for 0.5 h (Westali & Hesser, 1974). The hydrolysate was taken to dryness with a N_2 stream at 70 °C and resuspended in solvent B, and the amino acids were separated in solvent B. Appropriate standard amino acids in solvent B were cospotted, and the amino acids were visualized with ninhydrin.

Measurements of pH were made on a Beckman Century SS-1 pH meter that was standardized with phosphate NBS primary standard solutions at 23 °C (Bates, 1964). The electrode response was corrected for temperature. The observed pH was corrected for ionic strength by using $[H^+] = 10^{-pH/a_{H^+}}$ where a_{H^+} is the hydrogen ion activity coefficient at the appropriate ionic strength (Weast, 1970).

Buffers were prepared by mixing a solution of 0.01 M acidic component with a 0.01 M solution of the basic form of the buffer until the desired pH was obtained. The buffers used were as follows: $NaHPO_4$–Na_2PO_4, pH 6.00–8.09; $Na_2H_2P_2O_7$–$Na_3HP_2O_7$, pH 5.36–6.50; $Na_3HP_2O_7$–$Na_4P_2O_7$, pH 7.50–9.00. The ionic strength was varied with NaCl and was corrected to molality, m, by using $c = m\rho/(1 + mM)$, where c = [NaCl], ρ = solution density, and M is the molar mass of the solute (58.45 g). The density of NaCl solutions was

[1] Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonate anion; TNP, 2,4,6-trinitrophenyl; IOBA, *o*-iodosobenzoic acid; $[\text{melittin}]_0$, initial total concentration of melittin, regardless of ionization state; $[\text{TNBS}]_0$, initial concentration of TNBS; ami, amide; TLC, thin-layer chromatography; melittin_{x-y}, the peptide obtained from melittin containing the amino-terminal residue x and the carboxy-terminal residue y; TNP_x-melittin, ϵ-TNP-lysinyl$_x$-melittin; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; CD, circular dichroism; NMR, nuclear magnetic resonance.

Scheme I



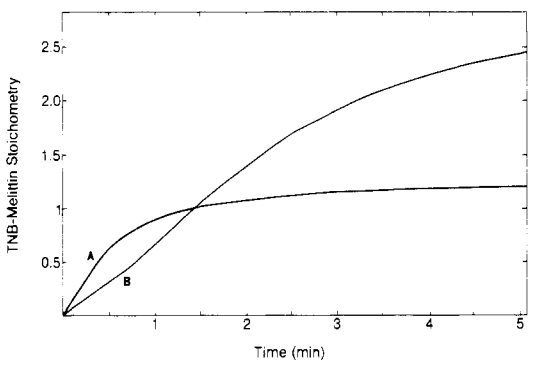


FIGURE 2: Reaction of 2,4,6-trinitrobenzenesulfonate with monomeric (A) and tetrameric (B) melittin. Reaction conditions: (A) $[melittin]_0 = 1.65 \times 10^{-5}$ M, $[TNBS]_0 = 4.27 \times 10^{-4}$ M, pH 8.51, $\Gamma/2$ 0.02, and 23 °C; (B) $[melittin]_0 = 1.88 \times 10^{-5}$ M, $[TNBS]_0 = 1.19 \times 10^{-3}$ M, pH 8.52, $\Gamma/2$ 2.43, and 23 °C. Molar absorptivity of TNP-lysine at 367 nm is assumed to be 1.05×10^4 cm^{-1} (Means et al., 1972).

taken from the *Handbook of Chemistry and Physics* (Weast, 1970). Ionic strength was determined from the calculated molality and the activity coefficients for various [NaCl] (Weast, 1970).

Results

The reaction of TNBS with amines has been extensively studied. The reaction mechanism suggested (Means et al., 1972; Scheme I) involves attack of the anionic TNBS on the unprotonated amine with the formation of a tetrahedral ring carbon transition state (Meisenheimer complex) in the rate-limiting step, followed by elimination of the thermodynamically favored HSO_3^- to form the substituted TNP-amino (I). Formation of II from I by ortho-directed bisulfite addition is favorable under most reaction conditions ($K_{HSO_3^-} = 3 \times 10^4$ M^{-1}; Means et al., 1972). I has a λ_{max} of 340 nm while II absorbs maximally at longer wavelengths (ca. 420 nm). For elimination of ambiguity in these kinetic experiments that might arise from the I $\rightleftarrows$ II equilibrium, the rate of product formation was monitored at 367 nm, an isosbestic point for I and II (Plapp et al., 1971). Figure 2 shows a typical time course for monomeric and tetrameric melittin. Of note is the increased reaction rate for monomeric melittin and the increased total extent of reaction of the tetramer. The slight hysteresis in the reaction course of TNBS with tetrameric melittin was an inconstant finding. The difference in total extent of reaction between monomeric and tetrameric melittin is a kinetic phenomenon; at t_∞ both reactions reach a stoichiometry for [TNBS]/[melittin] of 3.6 ± 0.2.

The reaction order was examined for both melittin and TNBS (Figure 3) under pseudo-first-order conditions and was completely explained by the simple second-order rate equation, $d[products]/dt = k_{obsd}[TNBS]_0[melittin]_0$; i.e., the linearity

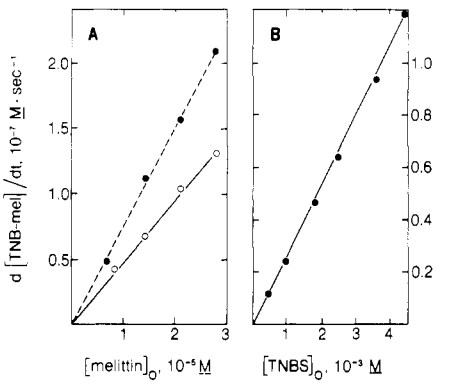


FIGURE 3: Determination of reaction order for TNP-melittin formation. Reaction conditions: sodium phosphate, 0.02 M, pH 7.12, 23 °C, and $\Gamma/2$ 0.05; (A) $[TNBS]_0 = 4.78 \times 10^{-4}$ M (○) and 9.50×10^{-4} M (●); (B) $[melittin]_0 = 2.47 \times 10^{-5}$ M.

of the plots of d[TNP–melittin]/dt vs. [X] for both melittin and TNBS indicates the reaction is first order in each, second-order overall. The linearity also indicates that a kinetically important melittin–TNBS noncovalent complex is not formed under these reaction conditions preceding the formation of the tetrahedral transition state since this would lead to a limiting value for d[TNP–melittin]/dt at high [melittin]; i.e., the reaction rate would become dependent on the concentration of the putative melittin–TNBS complex.

From Scheme I and the determined reaction order, we could imagine the observed reaction rate constant, k_{obsd}, to be related to k_1, k_{-1}, and k_2 in one of two ways. Either attack by RNH_2 (k_1) or breakdown of the tetrahedral ring carbon intermediate to products (k_2) could be rate limiting. In both cases, a simple second-order rate law would be obtained with k_{obsd} either equal to k_1 or $k_1k_2/(k_{-1} + k_2)$, respectively. Since an amine is a much poorer leaving group than the bisulfite ion (i.e., $k_{-1} \ll k_2$), the intermediate should breakdown almost exclusively by bisulfite elimination, in which case $k_{obsd} = k_1$.

Chemical Characterization of TNP-melittin. Before examining in detail the pH-dependent kinetics of TNBS reaction with melittin, we wished to determine the site or sites of reaction with TNBS. Melittin contains three lysine residues and an N-terminal glycine (Figure 1; Habermann, 1972) as potential reactants.

For determination of the site or sites of substitution in *monomeric* melittin, TNP-melittin, with a stoichiometry of TNP to melittin of about 0.3, was prepared in analytical quantities at pH 7.15, 23 °C, $\Gamma/2$ 0.01. This melittin adduct was analyzed as shown schematically in Figure 1. *o*-Iodosobenzoic acid cleavage of TNP-melittin resulted in two peptide fragments that could be separated by Sephadex LH-20 column chromatography (peptide I, R_f 0.44; peptide II, R_f 0.82). These separated peptides were subjected to hydrolysis with HCl–propionic acid and the free amino acids identified by TLC in solvent B. This analysis indicated, as expected for cleavage of tryptophanyl peptide bonds by IOBA (MaHoney et al., 1981), that peptide I is $melittin_{1-19}$ and that peptide II is $melittin_{20-26}$. The TNP moiety, which remained intact after IOBA treatment, was identified by its absorbance at 340 nm and was found entirely in peptide II; peptide I was devoid of absorbance at 340 nm. On the basis of the lower limit of spectroscopic sensitivity, the fractional molar content of TNP in peptide I (which means the fractional content of TNP-Lys-7 or TNP-Gly-1) is ≤0.03. Thus, TNP must be quantitatively substituted on Lys-21 and Lys-23.

Trypsin hydrolysis as depicted in Figure 1 was used to distinguish between TNP substitution of Lys-21 and Lys-23.

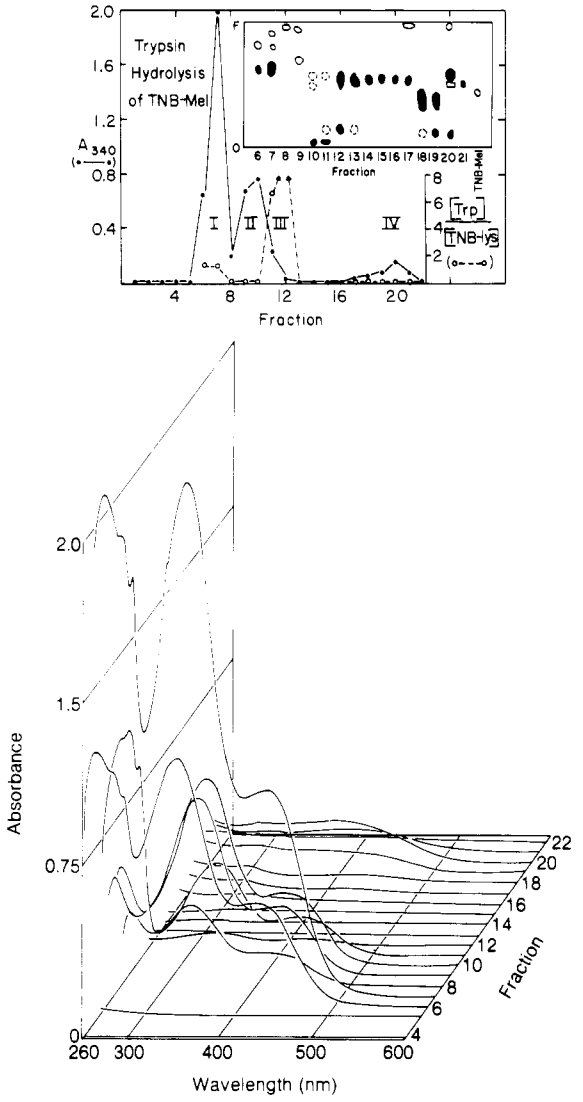


FIGURE 4: Structural characterization of TNP-melittin. TNP-melittin was formed under conditions in which melittin is monomeric (pH 7.15, $\Gamma/2$ 0.01). The TNP-melittin was purifed from the reaction mixture, partially cleaved with trypsin [trypsin/TNP-melittin (1:50 w/w), 3.5 h], and separated on a Sephadex LH-20 column in ethanol–formic acid (3:1 v/v). (Top) The elution profile, monitored at 340 nm, is shown as well as the [Trp]/[TNP-Lys] ratio, assuming extinction coefficients of 5570 cm^{-1} M^{-1} and 22 300 cm^{-1} M^{-1}, respectively. The inset shows the TLC pattern of each fraction on silica gel in solvent A. Closed shapes were visible only by ninhydrin spray while open shapes were yellow by visible light and blocked endogenous fluorescence, indicating the presence of TNP. Dotted shapes were weakly staining with ninhydrin. (Bottom) Spectra of fractions 4–22 from the Sephadex LH-20 column are shown from 260 to 600 nm. The reference cuvette contained ethanol–formic acid (3:1 v/v). Note the presence of tryptophan in fractions 6, 7, and 11, indicated by the characteristic absorption at 276 and 284 nm, and the absence of these features in fractions 8–10, which show only the 265-nm TNP adduct absorption.

The products of trypsin hydrolysis shown are those found for *unsubstituted* melittin; the nucleophilic substitution of an ϵ-amino of lysine by TNP renders that lysine resistant to trypsin action. Thus, if Lys-21 contained TNP, one would expect trypsin treatment to yield a single peptide containing both tryptophan and TNP (TNP_{21}-$melittin_{8-22}$), and one would expect to find a free lysine (Lys-23). If Lys-23 was substituted by TNP, trypsin treatment would yield a tryptophan-containing peptide devoid of TNP ($melittin_{8-21}$), and one would not find free lysine (since only Lys-23 is bridged by trypsin-sensitive residues).

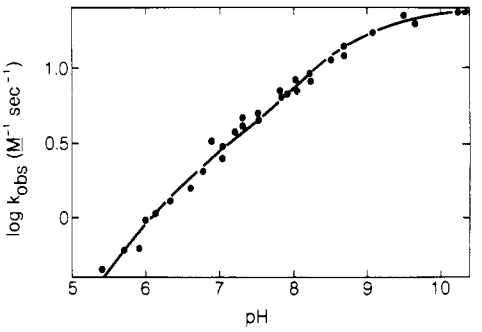


FIGURE 5: Reaction of TNBS with monomeric melittin. Reaction conditions: $[melittin]_0 = 1.65 \times 10^{-5}$ M, $[TNBS]_0 = 4.27 \times 10^{-4}$ M, $\Gamma/2$ 0.01, and $T = 23$ °C. The solid line was drawn for the scheme shown in eq 3 and 4 and the parameters for monomeric melittin.

Figure 4 shows the spectra, Sephadex LH-20 elution profile, and TLC pattern of a *partial* trypsin hydrolysis of TNP-melittin. All of the expected peptides from trypsin hydrolysis were separated by the combination of Sephadex LH-20 [ethanol–formic acid (3:1)] and TLC (solvent A). The amino acid content of these peptides was subsequently determined by acid hydrolysis and TLC in solvent B. Table I summarizes the structural data for these peptides. Both TNP-Lys-21 and TNP-Lys-23 are clearly present in TNP-melittin formed at pH 7.15, even at the relatively low TNP/melittin stoichiometry of 0.3.

Tetrameric melittin shows two kinetically rapid TNP adducts (Figure 2). TNP-melittin was prepared at $\Gamma/2$ 2.53 to a stoichiometry of 2.0 ± 0.2 in analytical quantities and cleaved with IOBA. The failure to identify TNP substitution in $melittin_{1-19}$ clearly indicates that the sites of TNP substitution in tetrameric melittin are Lys-21 and Lys-23.

Determination of Ionization Constants in Monomeric Melittin. From Scheme I, it is clear that the ionization of the amine, $RNH_2 + H^+ \rightleftharpoons RNH_3^+$, is important in determining the rate of the overall reaction, since the rate of nucleophilic attack on RNH_3^+ is negligible compared to the rate with RNH_2. If the rate equation $d[P]/dt = k_{obsd}[TNBS]_0[melittin]_0$ is rewritten to include only the un-ionized (i.e., reactive) fraction of $[melittin]_0$, one obtains

$$d[\text{TNP-melittin}]/dt = k_1[TNBS]_0[1/(1 + [H^+]/K_H)][melittin]_0 \quad (1)$$

Therefore

$$k_{obsd} = [1/(1 + [H^+]/K_H)]k_1 \quad (2)$$

If one studies the reaction as a function of pH, both K_H and k_1 can be obtained.

The monomeric state of melittin is thermodynamically favored by conditions of low concentration, ionic strength, and pH (Bello et al., 1982). At $\Gamma/2$ 0.01, melittin can be maintained as a monomer at ca. 10^{-5} M [melittin] to at least a pH value of 10.4. We examined the initial reaction rate of TNP-melittin formation under these conditions from pH 5.4 to 10.4 (Figure 5). We should emphasize that these data represent the initial rate of a *single* TNP substitution; additional substitution is kinetically slower in this pH range. As can be seen, the observed rate constant increased almost 100-fold between pH 5.5 and 10.0, indicating important changes in the ionization state of melittin in this pH range.

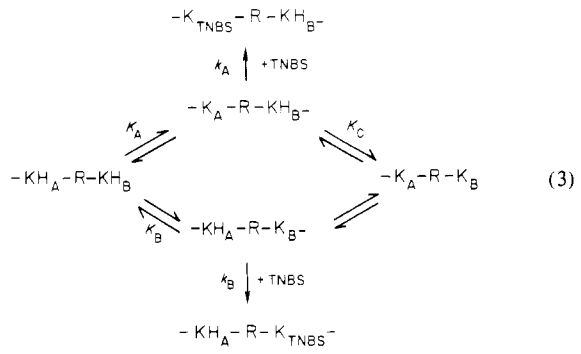
Any mechanism that proposes to describe the pH dependence of the reaction of monomeric melittin must include the experimental observation that both Lys-21 and Lys-23 form TNP adducts during the early course of the reaction (at $[\text{TNP-melittin}]/[melittin]_{total} \leq 0.3$) and yet the stoichiometry

Table I: Characterization of Peptide Sequences from Partial Trypsin Hydrolysis of TNBS–Melittin[a]

peptide column fraction no.	peak$_{fraction}$	TLC (R_f)	proposed sequence
1	I_7	0.89	G-X-G-A-V_5-L-K-V-L-T_{10}-T-G-L-P-A$_{15}$-L-X-S-W-X_{20}-K_T-R-
2	II_9	0.65	-K_T-R-Q_{25}-Q_{ami}
3	II_{10}	0.07	-K- R-Q_{25}-Q_{ami}
4	III_{12}	0.16	-V-X-T_{10}-T-G-X-P-A$_{15}$-X-I-S-W-X_{20}-K-
5	III_{12-17}	0.52	G-I-G-A-X_5-X-K-
6	IV_{20}	0.14	-R-, -K-, -K-R-
7	IV_{20}	0.50	-Q_{25}-Q_{ami}
8	IV_{20}	0.90	-K_T-R-
intact melittin			G-I- G-A-V_5-L-K-V-L-T_{10}-T-G-L-P-A$_{15}$-L-I- S-W-S_{20}-K- R-K- R-Q_{25}-Q_{ami}

[a] The symbol X indicates failure to identify a particular amino acid. The subscript T represents TNBS, and ami represents amide.

of the rapid phase of the reaction (Figure 2) is approximately 1. The simplest scheme that satisfies these criteria is one in which the ionization constants of Lys-21 and Lys-23 are linked, so that ionization to the free ϵ-amino form and subsequent nucleophilic substitution by TNP of one of these groups leads to perturbation of the ionization constant of the other, adjacent lysine. This is reasonable, since reaction of one of the lysine residues in the clustered basicity of the carboxy terminus of melittin would lower this basicity (by loss of a single positive charge) and thus raise the pK of the adjacent lysine and because the anionic bisulfite adduct of a TNP-lysine (II in Scheme I) would be expected to stabilize an adjacent, ionized lysine, also raising its pK value. Equation 3 shows such a



(3)

mechanism. In this formulation, the lysines designated A and B could be either Lys-21 or Lys-23. A reaction of TNBS with -K_{TNBS}-R-K_B-, -K_A-R-K_{TNBS}-, or -K_A-R-K_B- is not shown. This is a consequence of the above considerations about the perturbations of ionization constants. The pH dependence of k_{obsd} for eq 3 is

$$k_{obsd} = [1/(1 + [H^+]/K_A)]k_A + [1/(1 + [H^+]/K_B)]k_B \quad (4)$$

Using eq 4 and the values of $K_A = 10^{-6.5\pm0.2}$ M, $k_A = 3.1 \pm 0.3$ M^{-1} s^{-1}, $K_B = 10^{-8.6\pm0.2}$ M, and $k_B = 20 \pm 1$ M^{-1} s^{-1}, we generated the solid line in Figure 5.

Additional support for this interpretation can be obtained from the chemical identification of lysines A and B, through studies of the pH-dependent derivatization of Lys-21 and Lys-23. Specifically, the fractional derivatization at Lys$_B$ in eq 3 is given by

$$\frac{[\text{TNP-Lys}_B]}{[\text{TNP-Lys}]_{total}} = \frac{[\text{TNP-Lys}_B]}{[\text{TNP-Lys}_A + \text{TNP-Lys}_B]} = \frac{[1/(1 + [H^+]/K_B)]k_B}{[1/(1 + [H^+]/K_A)]k_A + [1/(1+ [H^+]/K_B)]k_B} \quad (5)$$

Complete trypsin hydrolysis permits the straightforward determination of the extent of TNP substitution of Lys-21 and Lys-23, since trypsin hydrolysis of TNP_{21}-melittin yields TNP_{21}-melittin$_{8-22}$ while that of TNP_{23}-melittin yields TNP-Lys$_{23}$-Arg$_{24}$. These peptides can be easily monitored by spectroscopy at 340 nm and can be separated by Sephadex LH-20 column chromatography (see Table I and Figures 4 and 6).

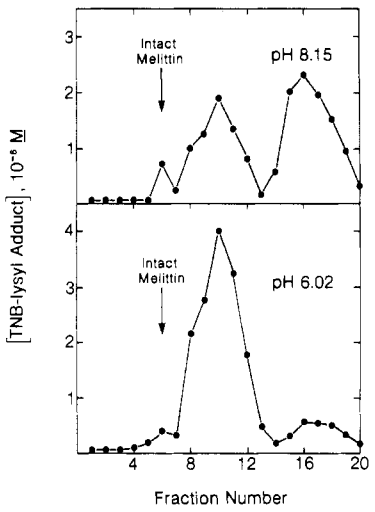


FIGURE 6: Sephadex LH-20 elution profile of TNP-melittin fragments from trypsin proteolysis. TNP-melittin was formed at pH 8.15 (top) or pH 6.02 (bottom), purified from the reaction mixture, treated with trypsin, and separated on a Sephadex LH-20 column under conditions described in detail in text and legend to Figure 4. The elution was monitored at 367 nm and the TNP-lysine adduct quantitated.

TNBS was reacted with monomeric melittin at several pH values for sufficient time to form a TNBS–melittin product with ≤0.4 TNBS groups/molecule. This low substitution stoichiometry assured that di-TNBS–melittin was not formed. The reaction was stopped by addition of ethanol–formic acid (3:1 v/v) to a final concentration of 5% to acidify the reaction solution. TNP-melittin was purified by Sephadex G-25 column chromatography, treated with trypsin for 18 h, dried down, resuspended in ethanol–formic acid, and separated on Sephadex LH-20. Two TNP-containing fragments were found at all pH values, corresponding to TNP_{21}-melittin$_{8-22}$ and TNP_{23}-melittin$_{23-24}$. Larger fragments, which could have arisen from partial hydrolysis products or from melittin with two TNP moieties, were not found. Figure 6 shows the chromatography profile at pH 6.02 and 8.15. As expected, the amount of each peptide differed with pH, with TNP_{23}-melittin$_{23-24}$ being more prevalent at pH 8.15. Since

$$[\text{TNP-Lys}_{21}]/[\text{TNP-Lys}_{21} + \text{TNP-Lys}_{23}] = [\text{peptide}_{8-22}]/[\text{peptide}_{8-22} + \text{peptide}_{23-24}] \quad (6)$$

and if one assumes that the extinction of TNP-Lys$_{21}$ = TNP-Lys$_{23}$, the fractional derivatization of lysine-21 can be

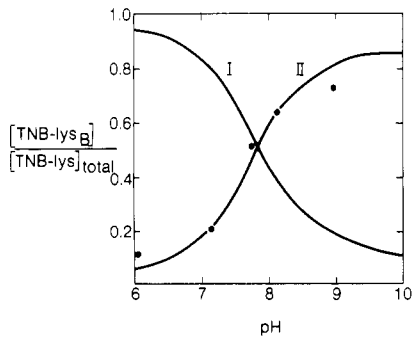


FIGURE 7: Dependence of fractional derivatization of lysine$_B$ on pH. TNP-melittin was formed as described in the text and legend to Figure 4 at the indicated pH values and the amount of TNP substitution at lysine-21 and -23 determined by using eq 5. The solid lines are theoretical curves, obtained from eq 5, assuming lysine$_B$ is lysine-21 (curve I) or lysine-23 (curve II).

easily determined from the A_{340} values of the column fractions. Figure 7 shows the data from these experiments. The solid lines represent the theoretical values for the fractional [TNP-Lys$_B$], obtained from eq 5 using the values of K_A, K_B, k_A, and k_B obtained above. Curve I is the expected result if lysine$_B$ is Lys-21 while curve II would be obtained if lysine$_B$ is Lys-23. Our data clearly identify lysine$_B$ as Lys-23, and lysine$_A$ as Lys-21.

The reaction of TNBS with tetrameric melittin is quite different from that with monomeric melittin since *two* TNBS molecules substitute in the kinetically rapid phase (Figure 2). At $\Gamma/2$ 2.54, melittin exists as a tetramer at [melittin]$_0 \geq 2 \times 10^{-6}$ M over an extended pH range (S. C. Quay and C. C. Condie, unpublished data). We examined the reaction rate, k_{obsd}, from pH 4.7 to 8.4 at $\Gamma/2$ 2.54 to determine the intrinsic reaction rate and ionization constants. These data (Figure 8) differ from those obtained with the monomer in two ways: the slope of the log k_{obsd}–pH curve between pH 5.8 and 7.4 is nearly unity (0.83) and there is a plateau below pH 5.6. Although not appreciated in Figure 6, the time course of the TNBS reaction with tetrameric melittin (Figure 2) clearly indicates the relatively rapid nucleophilic substitution of *two* TNBS molecules on melittin with further substitution occurring more slowly. This suggests that the ionization of Lys-21 and Lys-23 and the reaction of TNBS with the unionized form of these amino acids are *unlinked* and thus eq 7 and 8 describe the overall reaction. If we assume for

$$\begin{array}{ccc} \mathrm{LysH}_{21} & & \\ \uparrow\downarrow K_{21} & & \\ \mathrm{Lys}_{21} + \mathrm{TNBS} & \xrightarrow{k_{21}} & \mathrm{TNBS\text{-}Lys}_{21} \end{array} \quad (7)$$

$$\begin{array}{ccc} \mathrm{LysH}_{23} & & \\ \uparrow\downarrow K_{23} & & \\ \mathrm{Lys}_{23} + \mathrm{TNBS} & \xrightarrow{k_{23}} & \mathrm{TNBS\text{-}Lys}_{23} \end{array} \quad (8)$$

simplicity that $K_{21} = K_{23}$ and $k_{21} = k_{23}$, than the relationship of k_{obsd} to pH is

$$k_{obsd} = [2/(1 + [H^+]/K_{21})]k_{21} \quad (9)$$

Setting $k_{21} = 16.4$ M^{-1} s^{-1} and $K_{21} = 10^{-7.37}$ M adequately describes the reaction between pH 6 and 8.4. However, the reaction below pH 6 plateaus at a limiting value (which is not predicted by eq 9) and suggests a change in reaction mechanism in this pH range. This behavior for a TNBS reaction with amines has been observed before (Means et al., 1972) and has been attributed to a change in the rate-limiting step. One proposal for this change in mechanism is a role for general

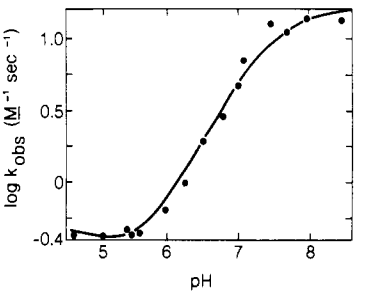


FIGURE 8: Reaction of TNBS with tetrameric melittin. Reaction conditions: [melittin]$_0 = 1.41 \times 10^{-5}$ M, [TNBS]$_0 = (1.19–2.37) \times 10^{-3}$ M, $\Gamma/2$ 2.54, and $T = 23$ °C. The solid line was calculated from eq 10 with $k_{HB} = 0.52$ M^{-1} s^{-1}, $k_{21} = 16.4$ M^{-1} s^{-1}, $K_{HB} = 10^{-5.3}$ M, and $K_2 = 10^{-7.37}$ M.

acid promotion of the breakdown of the intermediate Meisenheimer complex. This would permit the opportunity for a change in the rate-limiting step, from the formation of the Meisenheimer complex (Scheme 1; k_1) to an acid-catalyzed proton abstraction [see Means et al. (1972); thought to actually depend on the conjugate base of the appropriate acid]. Equation 10 contains such a term, with K_{HB} and k_{HB} being

$$k_{obsd} = [1/(1 + K_{HB}/[H^+])]k_{HB} + [2/(1 + [H^+]/K_{21})]k_{21} \quad (10)$$

the acid-catalyzed ionization constant and the rate-limiting step of this reaction, respectively. The solid line in Figure 8 was drawn from eq 10 and $K_{HB} = 10^{-5.3}$ M, $k_{HB} = 0.5$ M^{-1} s^{-1}, $K^{21} = 10^{-7.37}$ M, and $k_{21} = 16.4$ M^{-1} s^{-1}. The acid-catalyzed ionization constant obtained is close to the ionization constant of one of the buffer components (sodium pyrophosphate, p$K = 5.77$ at $\Gamma/2 \leq 0.1$; Weast, 1970), supporting the interpretation of the change in reaction mechanism put forward by Means et al. (1972).

Discussion

Because helix formation and self-association of melittin have been shown previously to depend on pH, ionic strength, and melittin concentration (Bello et al., 1982), the ionization constants of aminoacyl residues in melittin are of importance to our understanding of these conformational equilibria. We have determined the pK values for lysine-21 and -23 in melittin and have shown that these are abnormally low. Specifically, lysine-21 has a pK of 6.5 and lysine-23 a pK of 8.6 in monomeric melittin. In the tetramer these lysines have apparent pK values of 7.4.

Since, to the best of our knowledge, the largest perturbation of a lysyl ϵ-amino ionization constant is found in acetoacetic acid dehydrogenase (Schmidt & Westheimer, 1971), where the p$K_H = 5.9$, we wished to examine if the observed K_H values for melittin could be explained solely by electrostatic repulsion from the nearby arginine residues. X-ray diffraction studies of aqueously grown melittin cyrstals (Terwilliger et al., 1982) suggest that the carboxy-terminal portion of melittin is α helical. This would place Lys-21 approximately 0.56 nm from the unit positive charge (4.8×10^{-10} esu) of Arg-24 and about 0.91 nm from Arg-22. Setting the electrostatic potential for repulsion of two like point charges equal to the difference in free energy for dissociation of a 1:1 charge salt bridge, assigning K_H to the ϵ-amino of an unperturbed lysine ($10^{-10.53}$ M), and assigning K_{per} to the dissociation constant for Lys-21 in melittin ($10^{-6.5}$ M), we obtain

$$RT \ln \frac{K_{per}}{K_H} = \frac{q^2N}{Dr_1} + \frac{q^2N}{Dr_2} \quad (11)$$

where q is the electronic charge, N is Avogadro's number, D is the dielectric constant in the region of the charges, and r is the distance between the charges (r_1 = Lys-21 to Arg-22 and r_2 = Lys-21 to Arg-24). Solving eq 11 for D, one obtains a value of 17 for the effective dielectric constant in the region of Lys-21, Arg-22, and Arg-24. Since the value of the effective dielectric constant for intramolecular charge–charge interactions of organic molecules in aqueous solution can be much less than 15 (Westheimer & Kirkwood, 1938), the positive charges of Arg-22 and Arg-24 could account entirely for the Lys-21 pK of 6.0–6.5. Other factors of course could also contribute to the low pK of Lys-21, especially the hydrophobic interaction that could result from the close proximity of the indole ring of Trp-19 to the unprotonated ϵ-aminopentyl group of lysine (vide infra).

Bello et al. (1982) have examined the pH-, salt-, and concentration-dependent behavior of the CD spectra of melittin. These authors conclude that important titrations occur in melittin that affect hydrogen bonding and self-association in the pH range of 6–9.6. They obtained pK values ranging from 9.6 to ca. 6, depending upon the melittin concentration. These results are entirely consistent with ours, since in fact, their experiments were done under conditions in which the monomer–tetramer equilibrium position was changing. For example, they note that the pK of 9.6 is shifted to a lower pK value by high salt, an unexpected result from simple electrostatic considerations alone but consistent with the increase in tetramer formation accompanying the change in $\Gamma/2$.

Lauterwein et al. (1980) used proton NMR to determine that the α-amino of glycine had a pK of 7.8 in monomeric melittin. They found no other titrating groups from p^{2}H 3 to 9, in contrast to the work of Bello et al. (1982) and our own. Since the lysine CH_2 lines were assigned from p^{2}H titration shifts near p^{2}H 10, it is perhaps not surprising that ionizations between p^{2}H 6 and 9 were not observed. The use of salt free ^2H_2O for recording NMR spectra could have resulted in a shift of Lys-21 and -23 pK values even lower than the values obtained in this paper.

Terwilliger and co-workers (Terwilliger et al., 1982) determined the X-ray structure of tetrameric melittin and have provided a possible model of the lytic action of melittin. Their model of the interaction of melittin and bilayer membranes contains a structure for melittin that is identical with that found for the crystalline tetramer except that Lys-23 and Gln-26 were bent so that they do not extend into the lipid domain. This was apparently done to permit solvent access to Lys-23, which was presumably in a cationic state. Our results, which indicate a pK value of 7.4 for pK_{21} + pK_{23}, would permit placement of Lys-23 within the lipid domain as the extremely apolar, un-ionized aminoacyl residue with very little thermodynamic expense. This abnormally low pK value would also resolve the apparent conflict between the model of Terwilliger and co-workers (Terwilliger et al., 1982) and the high-resolution NMR work of Brown et al. (1982), in the placement of Lys-23. These NMR data clearly indicate that the ϵ-CH_2 of Lys-23 is close to the C_6 and C_7 protons of the indole ring of Trp-19, an unfavorable conformation if Lys-23 is protonated, since Trp-19 is on the apolar face of the melittin helix, but a quite reasonable finding if Lys-23 is, in fact, un-ionized.

The experiments described in this paper were performed at pH values well below the pK of an unperturbed lysine ϵ-amino group. The failure to find substitution of Lys-7 at the highest pH values suggests that this group has a more normal pK value. Assuming a 5% substitution of Lys-7 could have been detected, we can suggest that its pK_d is $\geq$9.6. The absence of adjacent ionizable amino acids to Lys-7 is consistent with these considerations. Similarly, the failure to detect kinetically significant quantities of TNBS substitution of the N-terminal glycine indicates the intrinsic rate constant for reaction with this group is less than 5% of that for Lys-21 or Lys-23. This may be due to significant steric hindrance by Ile-2 to TNBS attack. Nucleophilic substitution by TNBS is known to be very sensitive to steric conditions. For example, the intrinsic rate constants for TNBS reaction with aminocaproate (lysine analogue), glycine, glycylglycine, and 2-methylalanine are in the ratio 554:423:84:1 (Means et al., 1972).

Lewis & Shafer (1974) have elegently described the difficulty in assigning microscopic equilibrium constants to linked ionizations observed spectroscopically when the molar absorptivities of the pertinent groups are uncertain. In the present study, we have made the simplifying assumption that the molar absorptivities of TNP_{21}-melittin and TNP_{23}-melittin are equal and that ionization of adjacent unmodified lysine residues does not change these absorptivity values. Because it is likely that the anionic TNBS moiety can in fact form salt bridges with adjacent cationic lysine residues, it would not be unexpected that lysine ionizations could affect the absorptivity of the TNBS group. The absence of data about the magnitude of such effects, however, precludes their evaluation. Potentiometric titrations of melittin (S. C. Quay, unpublished experiments) obviates these difficulties and permits pK determinations independent of these ambiguities. These experiments suggest that the melittin lysine pK values obtained in this paper by TNBS reactivity are rather close to true protonic ionization constants.

Conclusion

The ionization constants for the lysine residues in the C-terminal portion of melittin have been determined. These pK values are between 6.5 and 8.6 for both aqueous states of melittin, monomeric and tetrameric. These pK values are 100–10 000 times lower than those expected for unperturbed ϵ-amino groups and well within the physiologically important pH range. This finding implies that the state of ionization of melittin can be altered by local pH and ionic strength, for example at a membrane surface, and permits the suggestion that these ionizations may have an important role in determining melittin–phospholipid interactions.

Registry No. 2,4,6-Trinitrobenzenesulfonic acid, 2508-19-2; melittin, 20449-79-0.

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Self-Association and Modification of Calcium Binding in Solubilized Sarcoplasmic Reticulum Adenosinetriphosphatase†

Jerson L. Silva and S. Verjovski-Almeida*

ABSTRACT: Solubilized sarcoplasmic reticulum adenosinetriphosphatase (ATPase) was fractionated by elution gel chromatography, in the presence of high concentrations of nonionic detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$) up to 74.4 mM (40 mg of $C_{12}E_8$/mL), using a wide range of ATPase concentrations. At 10 μg of ATPase/mL in the presence of 3.72 mM $C_{12}E_8$ (2 mg/mL) the enzyme eluted predominantly in the monomeric state, with a Stokes radius (R_s) of 45 Å. The ATPase in $C_{12}E_8$ had a tendency to self-associate when the protein was increased to 50 μg/mL or higher. The resulting aggregated particle had a R_s = 60 Å, compatible with a dimeric ATPase. The dimeric state was predominant at protein concentrations as high as 6.5 mg/mL, and larger aggregates were not present at a considerable proportion. Rechromatography indicated that monomeric and dimeric forms were in thermodynamic equilibrium. ATP (8 mM), when included in the elution buffer, shifted the equilibrium toward the monomer. In the presence of 50 μM $CaCl_2$, and in the absence of ATP, monomeric ATPase did bind 17.6 ± 1.5 nmol of Ca/mg of protein while the dimeric form did bind 9.4 ± 0.7 nmol of Ca/mg of protein. In vesicular ATPase, the maximal high-affinity binding was 9.5 nmol of Ca/mg of protein. Ca^{2+}-binding cooperativity was lost in the presence of 74.4 mM $C_{12}E_8$ in dimeric soluble ATPase. Ca^{2+} activation of ATP hydrolysis with soluble ATPase was noncooperative in the presence of 1 μM ATP, and cooperative in the presence of 5 mM ATP. It is apparent that a single polypeptide chain has two high-affinity Ca sites and that upon dimerization, half of the sites are not titrated. It is concluded that $C_{12}E_8$ promotes a loss in equilibrium binding cooperativity but does not prevent the Ca sites from interacting during the catalytic cycle upon addition of millimolar ATP. It is suggested that in vesicular ATPase the enzyme might predominantly be in a dimeric state and exhibit half-of-the-sites reactivity.

Sarcoplasmic reticulum adenosinetriphosphatase (ATPase) is a membrane-bound transport enzyme isolated from skeletal muscle in the microsomal fraction (Hasselbach, 1981). The enzyme can be solubilized by the nonionic detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] and retain its enzymatic activity (Dean & Tanford, 1978; LeMaire et al., 1978). The detergent replaces the membrane phospholipids and the ATPase becomes dispersed in the solution. Ultracentrifugation methods have been used to determine the molecular weight of the protein in solution. It was shown that with the use of $C_{12}E_8$ a monomeric from of the 117 000-dalton ATPase can be obtained (Dean & Tanford, 1978; LeMaire et al., 1978). However, the presence of higher molecular weight aggregates such as dimers, trimers, or tetramers simultaneously with the monomeric ATPase could not be ruled out by these authors. Dean & Tanford (1978) estimated that during sedimentation equilibrium measurements, up to 33% of the soluble ATPase could be in dimeric form and a concentration-dependent aggregation could be present. Another analytical method which has been applied to the characterization of the soluble ATPase is gel filtration chromatography (Dean & Tanford, 1978; LeMaire et al., 1978; Murphy et al., 1982). The Stokes radius of the soluble particle obtained by chromatographic measurements was 55–59 Å (Dean & Tandord, 1978; Murphy et al., 1982), a value which is higher than the 47 Å obtained by ultracentrifugation (Dean & Tanford, 1978). However, the protein concentrations used in gel filtration were 20–60 times higher than the ones used in ultracentrifugation experiments.

An important characteristic of the ATPase transport enzyme is its specificity for Ca^{2+}. The Ca^{2+}-transport ability of the enzyme cannot obviously be studied in the soluble ATPase because there are no compartments; however, Ca^{2+} binding and Ca^{2+} activation of ATP hydrolysis can be measured in the soluble preparation. It has been recently shown by Ca^{2+} titration of tryptophan fluorescence changes that upon solubilization of the ATPase by $C_{12}E_8$ the Ca^{2+} binding cooperativity is lost (Verjovski-Almeida & Silva, 1981). The stoi-

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[1] Abbreviations: $C_{12}E_8$, dodecyl octaethylene glycol monoether; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SR, sarcoplasmic reticulum; $NaDodSO_4$, sodium dodecyl sulfate.

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