Conformational Studies of Aqueous Melittin
CHARACTERISTICS OF A FLUORESCENT PROBE BINDING SITE*

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The structural basis of the interaction of melittin with amphipathic molecular assemblies, i.e. membranes, was investigated by studying the binding of 2-p-toluidinynaphthalelene-6-sulfonate (TNS) to melittin by ultraviolet and fluorescence spectroscopy. Monomeric melittin did not significantly bind TNS as judged by UV and fluorescence spectroscopy. Tetrameric melittin bound two TNS molecules per protomer with dissociation constants (Kd) of 4.2 x 10^-8 M. TNS binding to tetrameric melittin led to an increase in fluorescence quantum yield of 180-fold over the value for TNS alone in aqueous buffer (Φexp = 0.004). Five independent experimental findings suggest that the arginine residues of melittin provide one portion of the TNS binding site (presumably by formation of either a strongly stable "ringed-structure" salt bridge between the tetrahedral sulfonyl anion of TNS and the arginyl residues of melittin): 1) the Kd for binding is independent of pH from 6.0 to 10.8, the range in which the α-aminoxyeine and ε-aminoxyelines titrate; 2) TNS binding fails to perturb the kinetics of the reaction of 2,4,6-trinitrobenzenesulfonate with Lys-21 or Lys-23; 3) 1,7,21,23-acetyl-melittin, in which the NH2 terminus and all lysines are acetylated, binds TNS with a Kd similar to that for normal melittin; 4) guanidination of the NH2-terminal glycines and lysines of melittin (forming N1-guanidoglycine and homoarginyl residues, respectively) increases the number of TNS molecules bound per protomer to approximately 5; 5) conversion of Arg-22 and Arg-24 to the anionic N7,N18-(2,3-dihydro(7,7-dimethyl)bicyclo[2.2.1]heptane-1-methanesulfonyl)borate complex abolishes TNS binding, as judged by fluorescence.

Melittin undergoes significant inter- and intramolecular conformational changes as a function of ionic strength, pH, temperature, specific anion binding, and as a consequence of binding to membranes (Podo et al., 1982; Quay and Condie, 1983; Quay and Tronson, 1983). In our previous work, we have investigated the aqueous conformational dynamics by a variety of techniques. The information obtained in these studies is critical to the understanding of melittin-membrane interactions, since we now can dictate with quantitative precision the position of the monomer-tetramer self-association equilibrium as well as the COOH-terminal extended coil-helix transition.

As a preliminary investigation to the detailed study of membrane interactions, we have examined the binding to melittin of a fluorescent probe of protein surface environments (Edelman and McClure, 1968; Stryer, 1968; Chance, 1970; Slavik, 1982). Potassium 2-p-toluidinynaphthalelene-6-sulfonate has minimal fluorescence in water but shows a significant increase in fluorescence when bound to protein surfaces which provide a hydrophobic environment or which contain structural features which limit the mobility of solvent dipoles around the probe (Weber and Laurence, 1954; Slavik, 1982). Both fluorescence quantum yield and fluorescence emission λmax undergo dramatic changes upon protein binding. A careful investigation of TNS binding to peptides of the size of melittin (Beyer et al., 1972) indicated two structural requirements for these changes in fluorescence: an ionic interaction of the TNS anion with protein aminoacyl residues and an "aggregation" of the peptide-TNS complex which provides the necessary "hydrophobic or structured" environment.

In this paper we report that TNS binds to tetrameric melittin in a manner which enhances TNS fluorescence and that this binding requires the COOH-terminal arginine residues.

EXPERIMENTAL PROCEDURES

Materials

Potassium TNS and camphorquinone-10-sulfonic acid were obtained from Pierce and were used without further purification. Melittin, from Apis mellifera, was obtained from Sigma and was purified as described elsewhere (Quay and Condie, 1983). The melittin derivatives, 1,7,21,23-acetyl-melittin, 1-guanido-7,21,23-homoargininyl-melittin, and 22,24-N18,N18-(2,3-dihydro(7,7-dimethyl)bicyclo[2.2.1]heptane-1-methanesulfonyl)-melittin were synthesized and characterized in detail elsewhere. All other compounds were obtained from commercial sources.

Methods

Ultraviolet Spectra—These were recorded on a Hitachi 110 spectrophotometer at room temperature. Difference spectra were recorded using tandem cells. The reference cuvette contained a buffered solution (1.00 ml) of TNS in one side and a similarly buffered solution of melittin in the other side. The sample cuvette was prepared in an

1 The abbreviations used are: TNS, 2-p-toluidinynaphthalelene-6-sulfonate; Ac5-melittin, 1,7,21,23-acetyl-melittin; Gu4-melittin, 1-guanido-7,21,23-homoargininyl-melittin; DBH2-melittin, 22,24-N18,N18-(2,3-dihydro(7,7-dimethyl)bicyclo[2.2.1]heptane-1-methanesulfonyl)-melittin; ANS, 1-anilinonaphthalelene-8-sulfonate; Φ, fluorescence quantum yield.

2 S. C. Quay, manuscript in preparation.
identical fashion and the baseline was stored electronically and then recorded. The contents of the sample cuvette were then thoroughly mixed and the difference spectrum was recorded. Melittin concentrations were determined by the absorbance at 286 nm, assuming an extinction coefficient of 5570 M⁻¹ cm⁻¹. TNS concentrations were determined gravimetrically or estimated from the absorbance at 317 nm, assuming an extinction coefficient of 18,900 M⁻¹ cm⁻¹ (McClyre and Edelman, 1966).

Fluorescence Measurements—Measurements were made with a Perkin-Elmer Model MPF-44B fluorescence spectrometer. Fluorescence measurements were made at 20 °C in quartz cuvettes (10 × 10 mm or 2 × 10 mm). No correction of the emission spectra was made for the variation in sensitivity of the photomultiplier with wavelength. Fluorescence titrations of melittin with TNS were obtained by enhancement of TNS fluorescence (excitation wavelength 366 nm, emission wavelength 430 nm), by the quenching of the intrinsic fluorescence of Trp-19 in melittin (excitation wavelength 295 nm, emission wavelength 340 nm), or by singlet-singlet energy transfer from Trp-19 (donor) to TNS (acceptor) (excitation wavelength 295, emission wavelength 340 nm). The results were the same with all three techniques. Observed fluorescence intensities were corrected for dilution during the titration and, where necessary, for the inner filter effect. Relative quantum yields were estimated by comparison with a freshly prepared solution of quinine sulfate in 0.1 M H₂SO₄, assuming a quantum yield for quinine sulfate of 0.70 (Scott et al., 1970).

pH Values—These values were measured and buffers were prepared as described previously (Quay and Condie, 1983).

RESULTS AND DISCUSSION

Reaction of TNS with Monomeric Melittin—Melittin is monomeric at low concentrations and at low Γ/2 and pH (Quay and Condie, 1983). Initial experiments were performed to detect whether an interaction of TNS and monomeric melittin occurred, as judged by changes in TNS fluorescence quantum yield or emission λmax. No such changes were observed, even at the highest TNS and melittin concentrations used (pH 7.15; Γ/2 0.01; [TNS] = 1.16 × 10⁻⁴ M; [melittin] = 4.5 × 10⁻⁵ M). Since a 1% change in fluorescence emission could easily have been detected (because of the 180-fold increase in quantum yield which occurs upon binding, see below), the failure to detect any fluorescence change indicates that an interaction, if it exists, must have a Kd ≥ 10⁻⁹ M.

UV difference spectroscopy revealed slight hypochromic absorbance changes between 290 and 310 nm when TNS and monomeric melittin were in solution together, suggesting an interaction. These changes were similar to those reported for the interaction of TNS with linear tyrosine B (Beyer et al., 1972) and distinct from the absorbance changes which occur when a TNS-peptide interaction leads to TNS fluorescence quantum yield changes. The spectral changes were insufficiently dramatic to permit determination of a dissociation constant for the process. We can suggest the Ka for binding to monomeric melittin based on spectrophotometric changes is on the order of 10⁻⁹ M. We conclude from these studies that the interaction of monomeric melittin with TNS is weak, probably simply electrostatic in nature, and that this melittin-bound TNS is in an “aqueous”-like environment, without significant restriction of solvent dipole mobility.

Reaction of TNS with Tetrameric Melittin—The addition of TNS to solutions of tetrameric melittin (pH 7.20, 0.02 M sodium phosphate, Γ/2 0.63) leads to rapid and reversible changes in the fluorescence emission spectra of both TNS and melittin, suggesting a binding reaction. Fig. 1 shows records of the fluorescence emission from 300 nm to 475 nm (excitation = 280 nm) at [TNS]/[melittin] ratios of 0 (spectrum α) to 10 (spectrum k). The intrinsic fluorescence of the melittin tryptophan, centered near 335 nm, decreased as TNS was added, while the fluorescence intensity of TNS itself was enhanced. Under the same buffer conditions but in the absence of melittin, fluorescence emission of TNS cannot be detected with the instrumental configuration used to obtain the data in Fig. 1. Specifically, the relative quantum yield of bound TNS is 0.75, a 180-fold increase relative to the value in buffer alone (Φmax = 0.004).

The binding reaction of TNS with melittin and its thermodynamic parameters were determined by measuring the enhanced fluorescence of TNS when bound to melittin. Fig. 2 shows such a titration of melittin and indicates that 2 mol of TNS are bound per mol of melittin. Furthermore, the acetylation of melittin at Gly-1, Lys-7, Lys-21, and Lys-23 has relatively little effect on either the affinity or the stoichiometry of the reaction as can be seen from the curve in Fig. 2 for Ac₄-melittin. An Eadie-Scatchard plot (Eadie, 1942) of the binding data near the equivalence point can provide the dissociation constants of the TNS binding sites, with the usual assumptions about the binding behavior. For tetrameric melittin and for Ac₄-melittin, these Kd values are 4.2 ± 0.2 × 10⁻⁶ M and 5.1 ± 0.6 × 10⁻⁶ M, respectively.

Characterization of the TNS Binding Site—As indicated from the careful examination of TNS binding to peptides of the size of melittin (Beyer et al., 1970), the TNS-peptide binding interaction which leads to enhanced fluorescence quantum yield has two structural requirements: an ionic interaction between the TNS sulfonyl anion and a peptide or protein cationic site and a protein or peptide surface which can interact with the toluindinynaphthalene rings of TNS in a manner which restricts the mobility of solvent dipoles in the vicinity of the probe. The requirement for the latter site is consistent with the detailed x-ray diffraction study by Weber et al. (1979) of the ANS binding site of α-chymotrypsin. These authors showed that the ANS rings make contact primarily with polar amino acid residues on one side and with relatively ordered solvent water molecules on the other. These findings support the currently accepted solute-solvent interaction model of the photophysics of naphthalene probe fluorescence (Slavik, 1982, and references contained therein).
Fluorescent Probe Binding Sites in Melittin

FIG. 2. Fluorescent titration of melittin and Ac₄-melittin with TNS. Experimental conditions: same as in legend to Fig. 1 except: [melittin] = 2.1 × 10⁻² M; [Ac₄-melittin] = 2.2 × 10⁻² M; [TNS] as indicated. Excitation wavelength = 280 nm; emission wavelength = 450 nm; band widths = 6 nm.

Ionic Site—The potential sites for electrostatic bonding of TNS to melittin include the α-aminoglycine, 4 lysine residues, and 2 arginines. As we have already demonstrated, acetylation of Gly-1 and Lys-7, -21, and -23 does not appreciably change the stoichiometry of the interaction, suggesting that only the arginine residues are important for TNS binding. Additional support for this interpretation comes from four other kinds of experimental data.

1. We have shown previously (Quay and Tronson, 1983) that 2,4,6-trinitrobenzenesulfonate reacts rapidly with lysine-21 and lysine-23 near neutral pH. If TNS were to bind to these aminoacyl residues, the reaction with 2,4,6-trinitrobenzenesulfonate should be slowed considerably. In fact, under reaction conditions in which all of the tetrameric melittin is present in the form of a TNS complex (pH 7.5; 0.02 M sodium phosphate, 1/2 1.5; [TNS] = 9.6 × 10⁻³ M; [melittin] = 3.2 × 10⁻³ M), there was no detectable effect of TNS on the intrinsic rate constant or on the final stoichiometry of the reaction.

2. The $K_d$ for binding of TNS to tetrameric melittin was measured at seven different pH values from 6.04 to 10.8. Since the pK values for the α-aminoglycine and the three ε-aminolysines are expected to be within this pH range (lysine-21 and -23 have pK values near 7.4; Quay and Tronson, 1983), the TNS $K_d$ values should change with pH if these aminoacyl residues are involved in binding. In fact, no change in TNS binding was seen over this pH range.

3. O-Methylisourea can convert the α-aminoglycine and ε-lysines of melittin to guanidinium derivatives (specifically, N-guanidoglycine and homoarginine). We prepared this derivative under standard reaction conditions (Habeeb, 1972). This melittin derivative, which now has six potential "arginine-like" aminoacyl residues, binds TNS well. Fig. 3 shows a Hill plot of the binding of TNS to this derivative and suggests that tetrameric Gu₄-melittin has a number of binding sites, $n$, for TNS which interact in a cooperative manner. From the maximum slope ($n_H = 4.5$), one can conclude that there are at least five TNS binding sites on Gu₄-melittin, since $n$ must be $\geq n_H$ (Segal, 1975).

4. Finally, arginine-selective reagents, especially of the diketone class (Yankeelov, 1972), should yield melittin derivatives which do not bind TNS if the arginine residues are critical for this process. One such reagent, camphorquinone-10-sulfonic acid, is highly specific for arginine residues and forms stable adducts under mild reaction conditions (Pande et al., 1980). These derivatives are anionic in borate buffers due to the addition of borate to the dipolar arginine adducts (Riordan, 1973). We prepared this derivative of melittin under the reaction conditions specified by Pande et al. (1980) and examined this derivative for TNS binding. We found no enhancement of TNS fluorescence under the conditions we tested for fluorescence ([TNS] = 2.1 × 10⁻⁴ M; [DBH₂-melittin] = 6 × 10⁻⁵ M) and thus conclude that if binding occurs at all it is with a dissociation constant greater than about 5 mM. The failure of this melittin derivative to bind TNS could be due either to steric hindrance by the bulky arginine substituents, preventing access to the hydrophobic or polar peptide surface site for TNS, or more likely, to the inability of this derivative to form the primary TNS-melittin bond, the ionic salt bridge.

Relationship of TNS Binding Site to the Crystallographic Structure of Tetrameric Melittin—Terwilliger and Eisenberg (1982) have recently completed the x-ray crystallographic structure of melittin at 2-Å resolution. Although their data were obtained with tetrameric melittin crystals grown under very high ionic strength conditions and thus may not correspond in detail with the tetramer structure, it would nonetheless be useful to examine TNS binding to melittin in light of their findings. They found that the arginine residues of melittin form one part of the wall of a solvent-containing cavity near the COOH terminus of melittin. Trp-19 forms the base of this cavity and is "in close contact with many apolar side chains" (Fig. 3; Terwilliger and Eisenberg, 1982). This cavity is ideally placed to accept the sulfonfonyl anion and naphthalene ring of TNS, although some adjustment of the melittin side chains to increase the cavity volume might be necessary to fully accommodate the naphthalene rings. The ordered solvent within the cavity is ideally suited to provide the necessary solvent-solute interactions which promote TNS fluorescence. The binding of TNS to tetrameric concanavalin A has also been proposed to be located in the cleft between subunits (Yang et al., 1974). In the case of concanavalin A, one should note that the subunits are over 9 times larger than those in melittin.

Fig. 3. Hill plot of the binding of TNS to tetrameric Gu₄-melittin. TNS fluorescence was measured as indicated in the legend to Fig. 2.
Specificity of Arginine for TNS Binding—The specificity for arginine as the cationic portion of the TNS-melittin salt bridge is striking. A priori considerations might lead to the supposition that the α-amino and/or ε-amino groups might also serve such a role. In the case of the cyclic tyrocidin decapetides, the ε-amino group of ornithine is clearly important for TNS binding since succinylation of this residue blocked all TNS interactions (Beyer et al., 1972).

On the other hand, there is considerable precedent for the binding of anions with tetrahedral structures to protein arginine residues (Ichimura et al., 1978; Mita et al., 1978 and 1980). For example, histone-DNA interactions, in which the histone arginine residues are responsible for binding to DNA phosphate anions, is well characterized (Ichimura et al., 1982). Similar salt bridges between PO₄⁻ and the arginine guanidinium group have been identified by x-ray crystallography of staphylococcal nuclease and have been proposed to explain the mechanism of action of the enzyme (Cotton et al., 1979).

In the case of melittin, we propose this “ringed-structure” salt bridge to represent the molecular structure of the TNS-melittin electrostatic bond. Melittin has been shown to bind other tetrahedral anions with similar specificity and to undergo conformational changes as a consequence of this binding (Podo et al., 1982).

What Is the Relationship of TNS-Melittin Interactions to Phospholipid-Melittin Interactions?—Melittin has a well known propensity to interact strongly with phospholipids, causing permeability changes of bilayer membranes (Tosteson and Tosteson, 1981) and cellular lysis (Habermann, 1972). It thus seemed appropriate to question whether TNS binding to melittin is a suitable model for the more complex interactions with bilayer lipids. The answer is mixed. Some features are clearly similar, for example, the amphipathic nature of all three compounds. However, melittin can clearly bind to lipids in a monomeric state (and, in fact, we feel that red cell lysis is initiated largely by the monomer) while TNS binding and fluorescence occurs predominantly with the tetramer. The binding of TNS to monomeric melittin is considerably weaker than to tetrameric melittin and lacks the requisite features to support the TNS fluorescence quantum yield changes observed with the tetramer. These features include a need for relatively rigid solvent dipoles, a feature lacking in the predominantly random-coil structure of the melittin monomer (Podo et al., 1982).

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