Matkő, J., Trőn, L., Balázs, M., Hevessy, J., Somogyi, B., & Damjanovich, S. (1980) *Biochemistry 19*, 5782.
McCammon, J. A., & Karplus, M. (1980) *Annu. Rev. Phys. Chem. 31*, 29.
Means, G. E., & Feeney, R. E. (1971) in *Chemical Modification of Proteins*, pp 132–134, Holden-Day, San Francisco, CA.
Nakanishi, M., Tsuboi, M., & Ikegami, A. (1973) *J. Mol. Biol. 75*, 673.
Pongs, O. (1970) *Biochemistry 9*, 2316.
Sober, H. A. (1970) in *CRC Handbook of Biochemistry*, B74–77, Chemical Rubber Publishing Co., Cleveland, OH.
Somogyi, B., & Damjanovich, S. (1971) *Acta Biochim. Biophys. Acad. Sci. Hung. 6*, 353.
Somogyi, B., & Damjanovich, S. (1975) *J. Theor. Biol. 51*, 393.
Staerk, H., & Chance, B. (1969) *FEBS Lett. 3*, 287.
Steinberg, I. Z. (1971) *Annu. Rev. Biochem. 40*, 83.
Stryer, L., Thomas, D. D., & Meares, C. F. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 203.
Takahashi, K. (1961) *J. Biochem. (Tokyo) 49*, 1.
Takahashi, K. (1965) *J. Biol. Chem. 240*, PC4117.
Uchida, T., & Egami, F. (1967) *Methods Enzymol. 12*, 228–239.
Welch, G. R., Somogyi, B., & Damjanovich, S. (1982) *Prog. Biophys. Mol. Biol. 39*, 109.
Woodward, C., Simon, I., & Tüchsen, E. (1982) *Mol. Cell. Biochem. 48*, 135.

Effect of Pressure on the Self-Association of Melittin†

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ABSTRACT: The effect of increased hydrostatic pressure (1 bar to 1.8 kbar) on the self-association of melittin was measured by using the fluorescence anisotropy of its single tryptophan residue. The degree of self-association was found to decrease with increasing pressure. The volume change (ΔV) for dissociation is surprisingly large. At low pressures, ΔV for dissociation is near −150 mL/mol. The magnitude of the volume change decreased with increasing pressure, possibly as a result of pressure-induced compression of free volume trapped at the subunit interface region of the tetramer. Overall, the pressure-dependent association of melittin is comparable to that expected for hydrophobic interactions and to that found for micelle formation by detergents.

Melittin is an amphipathic peptide component of bee venom which enhances the venom phospholipase activity and disrupts phospholipid bilayers. It has been shown to interact strongly with phospholipid bilayers, in some cases causing cell lysis (Dufourcq & Faucon, 1977; Mollay & Kriel, 1973; Habermann, 1972, 1980; Sessa et al., 1969). It has thus served as a useful model system for studies of lipid–protein interactions. Melittin exists in two forms in solution, as a monomer and as a tetramer (Talbot et al., 1979). In this report, we describe the effects of increased hydrostatic pressure (1 bar to 2 kbar) on the self-association of melittin.

We measured melittin's self-association by fluorescence spectroscopy. As with other optical methods, it is convenient for observing pressurized samples. Also, it is sensitive enough to be useful at the low protein concentrations where association is incomplete, and the greatest effects of pressure perturbation may be seen (Weber & Drickamer, 1983). Melittin has several properties which make its self-association easily measurable by fluorescence techniques. Each peptide chain contains a single tryptophan residue and no tyrosine residues (Habermann & Jentsch, 1967). Hence, the intrinsic fluorophore population is likely to be homogeneous, and there is no possibility of tyrosine–tryptophan energy transfer. Upon association to form the tetramer, the emission of the protein shifts from 353 to 337 nm and the anisotropy approximately doubles (Talbot et al., 1979; Faucon et al., 1979). The blue shift of the tryptophan emission in the tetramer occurs because the tryptophan residues are shielded from contact with the solvent (Terwilliger & Eisenberg, 1982). The large change in anisotropy occurs because the rotational correlation times of the monomers (1.2 ns) and tetramer (3.8 ns) are substantially different, and because both are comparable to the fluorescence lifetime of the tryptophan residue (≈2.5 ns) (Lakowicz et al., 1983; Georghiou et al., 1981). In addition, the segmental mobility of the tryptophan residue may be less in the tetramer than in the monomer. Thus, we measured the self-association of melittin by measuring the fluorescence anisotropy of its intrinsic tryptophan. Finally, for confirmation, we also measured the self-association of melittin labeled with an extrinsic probe, *N*-methylisatoic anhydride, and obtained similar results.

The recently reported crystal structure is informative with respect to the molecular interactions which influence melittin self-association (Terwilliger & Eisenberg, 1982). The tetrameric structure is not stabilized by either hydrogen bonds or electrostatic interactions. The latter fact is not surprising given that melittin lacks even a single negative charge, but the lack of hydrogen bonding between the subunits clearly makes melittin an unusual case. As Eisenberg and his colleagues have pointed out, the sole interaction that promotes the tetramerization reaction is hydrophobic. Thus, it is similar to the formation of micelles by amphipathic molecules such as detergents. Melittin monomers are grossly similar to such amphipathic molecules, having hydrophobic residues concentrated at one end of the peptide chain and basic residues grouped

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0006-2960/84/0423-3411$01.50/0

together at the carboxyl terminus. Melittin shares another property with amphipathic molecules in that its self-association is promoted by the presence of counterions which minimize the repulsive interactions of the like charges. In particular, tetramer formation is not observed even at very high melittin concentrations in the absence of salt, and conversely, melittin seems to be present wholly as the tetramer if the salt concentration is above 2 M. Experimentally this is a very useful property, since it allows the degree of monomer association and the protein concentration to be varied independently. Thus, we see that melittin provides a unique yet very accessible instance of protein association.

Recently, the effects of high pressure on biomolecules have been the focus of much interest (Weber & Drickamer, 1983; Jaenicke, 1981; Offen, 1980). There are several reasons for this. First, such studies are relevant to understanding barophilic organisms of the ocean depths, the pressure-induced reversibility of anesthesia (Trudell et al., 1973), and the effects of pressure on divers. Also, pressure is a relatively gentle and typically reversible perturbation. Moreover, in the pressure range utilized in this study, approximately 1–2000 atm, covalent bonds are neither bent nor broken. Rather, pressure in this range seems to affect mostly those weak interactions such as hydrogen bonds, hydrophobic "bonds", and salt bridges which play large roles in the structure of biological molecules. Finally, the effects of pressure are (potentially) explainable using thermodynamic theory.

While the effects of pressure on protein structure have been studied by several investigators (Heremans, 1982; Weber & Drickamer, 1983; Zipp & Kauzmann, 1973; Chryssomallis et al., 1981; Brandts et al., 1970; Suzuki, 1972), only one systematic study by direct observation has been undertaken of the effects of pressure on protein self-association, that of Paladini & Weber (1981a). This milestone paper describes experiments similar to our own, but on a dimeric protein, enolase. They found that pressure promoted dissociation and described a useful model which predicted this result. This prediction was based upon the hypothesis that free volume is trapped at the interface between protein subunits. We note that several groups [see Harrington & Kegeles (1973), Jaenicke (1981), and Heremans (1982) and references cited therein] have indirectly studied association under pressure by observing subunit scrambling after depressurization. Melittin is significantly different from enolase and other previously studied proteins because salt promotes rather than hinders its self-association, its intrinsic tryptophans are a homogeneous population of fluorophores that provide both a useful spectral shift and a polarization change upon association, and there seems to be no specific effect of divalent cations. Finally, while for enolase several sorts of interactions may contribute to intersubunit binding, in melittin only hydrophobic interactions are responsible for self-association. Thus, melittin serves as a special case for assessing the importance of this interaction in protein structure and the effect of pressure on hydrophobic interactions in proteins.

Theory

Our theory for the melittin self-association is similar to that of Paladini & Weber (1981a) except that we consider here the formation of a tetramer. Thus, the dissociation constant is

$$K_D = [M]^4/[T] \tag{1}$$

where M and T are monomer and tetramer, respectively. If we represent the total protein concentration (as tetramer) as C_0 and define α, the degree of dissociation, to be equal to 1 when dissociation is complete, we obtain

$$[M] = \alpha 4C_0 \tag{2a}$$

and

$$[T] = (1 - \alpha)C_0 \tag{2b}$$

Redefining K_D in these terms allows us to compute the pressure derivative of ln K_D, which is a function of the volume change (ΔV) for the reaction:

$$\frac{d \ln K_D}{dP} = \frac{-\Delta V}{RT} = \frac{4 - 3\alpha}{\alpha(1 - \alpha)} \frac{d\alpha}{dP} \tag{3}$$

where R is the gas constant and T the absolute temperature. A negative value for ΔV will be obtained if pressure shifts the equilibrium toward increased amounts of monomer. We obtain α from the anisotropies (or average emission wavelengths) as described by Paladini & Weber (1981a):

$$\alpha = \left(1 + Q^{-1}\frac{r - r_M}{r_T - r}\right)^{-1} \tag{4}$$

where Q is the ratio of the tetramer quantum yield to that of the monomer and r_M, r_T, and r are the anisotropies of the monomer, tetramer, and the sample, respectively, each as a function of pressure. For clarity, we note that the anisotropy values used in eq 4 are the pressure-dependent values of r_M, r_T, and r. Similarly, Q is also determined at each pressure. In this way, the calculated values of α are not affected by the effects of pressure on the anisotropy or quantum yield of the monomer or the tetramer. Rearranging eq 3 and integrating over the same range of α and pressure, we obtain

$$\ln K_D = \ln [\alpha^4/(1 - \alpha)] = \frac{-P\Delta V}{RT} + C \tag{5}$$

A plot of ln $[\alpha^4/(1 - \alpha)]$ vs. P yields a curve whose slope at any pressure is ΔV for the reaction at that pressure. Finally, we note that ΔV is also the slope of the curve of ΔG for the reaction, plotted vs. pressure:

$$\left(\frac{\partial \Delta G}{\partial P}\right)_T = \Delta V \tag{6}$$

Thus, ln $[\alpha^4/(1 - \alpha)]$ is a linear function of ΔG for the reaction; 1 kcal/mol is equivalent to 1.69 on the ln $[\alpha^4/(1 - \alpha)]$ scale; this quantity is indicated in Figure 4.

Materials and Methods

Melittin was from Sigma, was determined to be homogeneous by disc gel electrophoresis, and was used without further purification. Samples were prepared for each experiment by diluting a concentrated stock solution of the peptide into 10 mM pH 7.5 tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer containing NaCl of various molal concentrations. The pK_a of Tris is known to be largely pressure insensitive in this range (Neuman et al., 1973). Protein concentrations are expressed as the monomer.

The samples were pressurized in a high-pressure fluorescence vessel (bomb) essentially identical with the one used by Paladini & Weber (1981a,b). Fluorescence phase and modulation lifetimes, spectra, and anisotropies were measured on SLM instruments as previously described (Paladini & Weber, 1981a; Lakowicz & Thompson, 1983). In particular, anisotropies were measured with a 4-nm excitation band-pass centered at 300 nm and passed through a Corning 7-54 filter; the emission was monitored through Corning 0-52 and 4-69 filters at the zero order of the emission monochromator. The

anisotropies were corrected for the depolarization caused by pressure-induced birefringence of the windows by measuring the apparent anisotropy of *N*-acetyltryptophanamide in vitrified propylene glycol at −58 °C. It was found that this compound gave a more accurate correction than fluorescein in glycerol; the reason for this remains unclear. Spectra were not corrected but were obtained under identical conditions for each experiment. At 25 °C, the viscosity of pure water is known to change by just over 2% in this pressure range (Bridgman, 1926; Horne & Johnson, 1966). However, the influence of high concentrations of salt on the pressure dependence of this parameter remains virtually unknown (Sergeevich et al., 1953). We decided to assess the change in the pressure dependence of the viscosity of the various salt solutions by the fluorescence anisotropy method. In particular, we selected fluorescein-labeled lysozyme, whose emission anisotropy is known to accurately reflect the viscosity of dilute aqueous solutions to at least 5 kbar (Chryssomallis et al., 1981), and measured its anisotropy as a function of pressure in 0, 0.5, and 2.0 *m* solutions of sodium chloride in Tris buffer. We found no apparent difference in the pressure dependence of viscosity of these solutions; we note that the method is not accurate to more than a few percent.

The effect of pressure on the apparent intensities of the monomer and tetramer, due to both solvent compression and quenching, was corrected for by monitoring the total intensity from the peptide in both 0.0 and 2.0 *m* NaCl as a function of pressure. We found that the intensity of the monomer stays constant with pressure but that the tetramer intensity decreases linearly by ~5% in 1800 bar; these results are in agreement with our measurements of phase and modulation fluorescence lifetimes (results not shown). These small, pressure-dependent changes in relative quantum yield were incorporated as pressure-dependent values of Q in eq 4; Q at 1 atm was 0.90.

We also measured the self-association under pressure of melittin labeled with an extrinsic fluorescence probe, *N*-methylanthranilate. The modified melittin was synthesized by reaction of the protein with *N*-methylisatoic anhydride; details of the procedure and characterization of the product will appear elsewhere (A. Hermetter and J. R. Lakowicz, unpublished results). The labeled melittin behaves almost identically with unlabeled protein, exhibiting an increase in fluorescence anisotropy upon formation of the tetramer, and a similar salt effect on the self-association. Thus, we measured the self-association of the derivative by using the same fluorescence anisotropy method described above, but with different spectral conditions. Samples having a 1:50 labeled:unlabeled ratio were excited at 355 nm through a Corning 7-54 filter and their emissions observed through Corning 3-74 and 4-69 filters. Pressure-dependent anisotropies of monomer, tetramer, and the experimental case were measured, and α was calculated by using eq 4. Values for the birefringence correction were obtained from the pressure-dependent anisotropies of the methylamide of *N*-methylanthranilate in glycerol at −70 °C. The ratio Q was equal to 1 at 1 atm and did not appear to vary with pressure.

Results

The pressure-dependent anisotropies of melittin at various protein and NaCl concentrations are depicted in Figure 1. The anisotropies were corrected for pressure-induced birefringence as described under Materials and Methods. We expect samples with higher protein and salt concentrations to exist more in the tetramer form and hence exhibit higher anisotropies (Talbot et al., 1979; Faucon et al., 1979). This is clearly the case, as the values of 1 atm show. Samples in

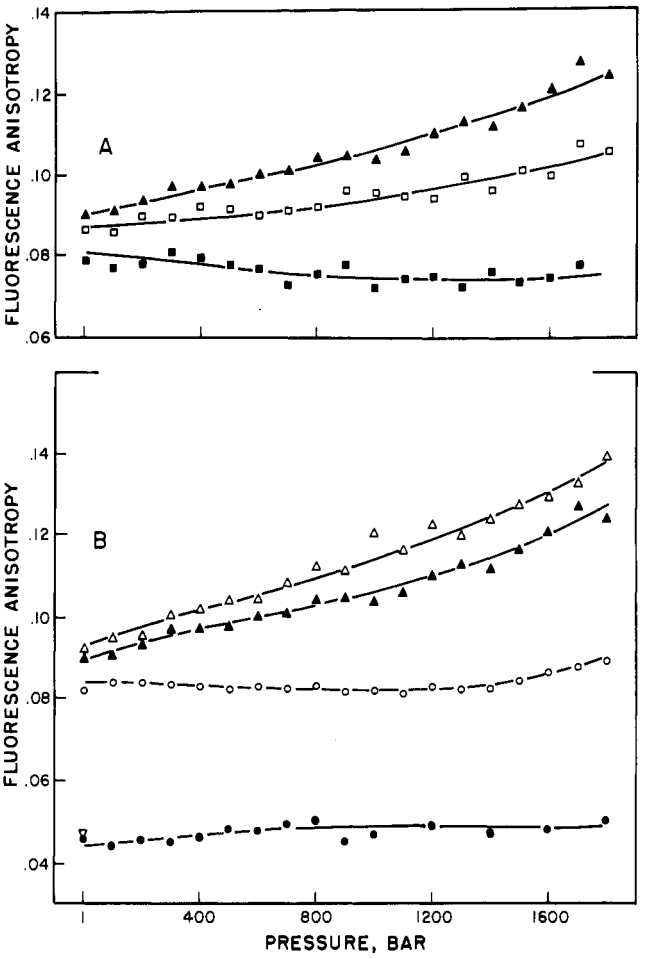

FIGURE 1: Effect of pressure on the fluorescence anisotropy of melittin at selected concentrations of melittin and NaCl. Anisotropies were corrected for the pressure-induced birefringence of the pressure cell windows. (A) The melittin concentrations were 200 (▲), 50 (□), and 10 (■) μM. [NaCl] = 1.0 *m*. (B) The melittin concentration was 200 μM. The concentrations of NaCl were 2.0 (△), 1.0 (▲), 0.5 (○), and 0.0 (●) *m*. The inverted triangle (▽) indicates the anisotropy after returning to 1 atm.

no salt and 2 *m* salt exhibit a very modest rise and a dramatic increase in anisotropy with pressure, respectively. At intermediate salt concentrations, the increase in anisotropy is also intermediate.

Several trivial causes may be suggested for these changes in anisotropy. One possibility is a change with pressure of the relative energies or populations of the ^1L_A and ^1L_B excited states of the tryptophan, and thus its excitation polarization spectrum. At our excitation wavelength, 300 nm, the absorption is dominated by that of the ^1L_A state (Valeur & Weber, 1977), and a small shift is not likely to cause a substantial change in the anisotropy. To further discount this possibility, we examined *N*-acetyl-L-tryptophanamide in propylene glycol at −58 °C. We found no change in its excitation polarization spectrum in this pressure range. Moreover, no changes in anisotropy attributable to absorption spectral shifts have been observed in other proteins over this pressure range (Paladini & Weber, 1981a; Chryssomallis et al., 1981). Other fluorescence parameters of substituted indoles do exhibit modest changes (Hook & Drickamer, 1981; Politis & Drickamer, 1981). The clearest evidence against such a shift is that the anisotropies of melittin at different concentrations in the same (intermediate) concentration of salt show overtly different pressure dependencies (Figure 1A). This fact also negates the possibility that differing pressure–viscosity coefficients of the salt solutions are the source of the changes in

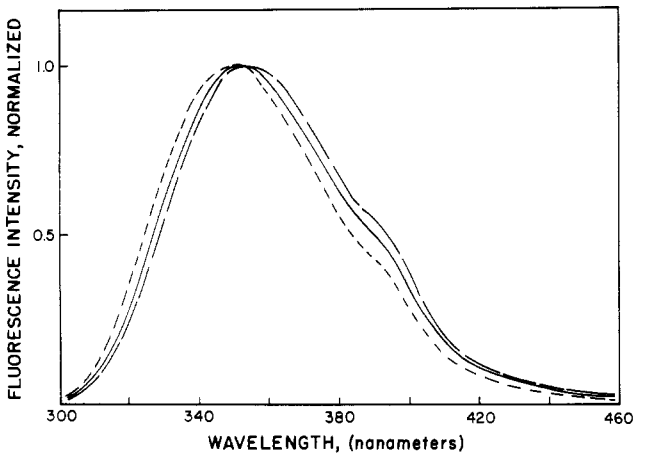

FIGURE 2: Fluorescence emission spectra of 50 μM melittin in 1.0 *m* NaCl. The pressures were 1 atm (---), 600 bar (—), and 1800 bar (--).

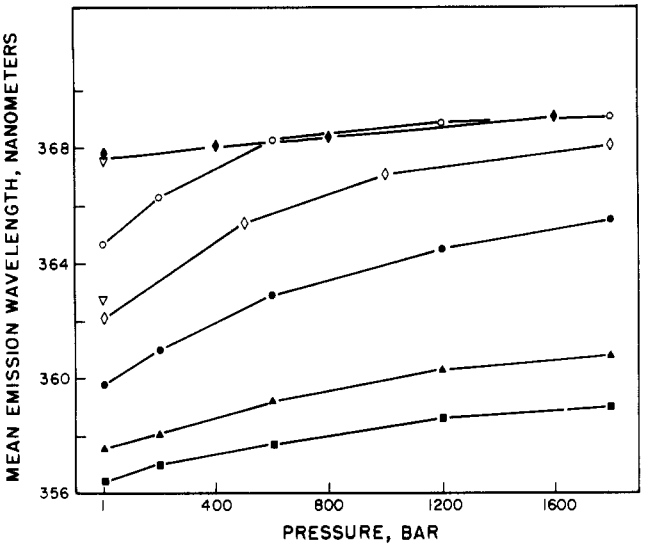

FIGURE 3: Average wavelength of fluorescence emission as a function of pressure for melittin at selected concentrations of melittin and NaCl. At [melittin] = 200 μM, the concentrations of NaCl were 0.0 (♦), 0.5 (●), 1.0 (▲), and 2.0 (■) *m*. At 0.5 *m* NaCl, the concentrations of melittin were 200 (●), 50 (◊), and 20 (○) μM. The inverted triangles indicate the anisotropies after returning to 1 atm.

anisotropy. As described under Materials and Methods, the pressure-dependent viscosities of salt solutions from 0 to 2 *m* are equivalent to within our experimental error of a few percent.

The fact that there is a dependence on protein concentration of these anisotropies (Figure 1A) also negates any suggestion that conformational changes and lifetime variations could fully account for our data. However, the 50% increase in anisotropy of the melittin at high protein and salt concentrations (Figure 1B) is much greater than can be attributed to the ~0.5-ns decrease in lifetime (results not shown) or the 2% increase in viscosity. The tryptophans in the melittin tetramer are known to have substantial freedom of motion (Georghiou et al., 1981; Lakowicz et al., 1983), and hence, we feel that the simplest explanation for this observation is that the segmental mobility of the tryptophans in the tetramer is decreased by pressure. In comparison, the protein in salt-free solution (monomer) exhibits a very slight increase in anisotropy, which is attributable to solvent viscosity since the lifetime remains essentially constant.

We also obtained fluorescence emission spectra of melittin under the same conditions; those of 50 μM melittin in 1.0 *m* NaCl are depicted in Figure 2. The well-known (Talbot et al., 1979) red-shifted emission of the melittin monomer with respect to the tetramer leads one to conclude that pressure causes the tetramer to dissociate. Similar (though usually less shifted) spectra were obtained for melittin as a function of pressure in the same experiments that produced the data in Figure 1. The average emission wavelengths at various pressures are summarized in Figure 3. For the same reasons cited above, the best explanation for these spectral data is that pressure promotes dissociation of melittin.

We propose that melittin in 2.0 and 0.0 *m* NaCl solutions remains in the tetramer and monomer states, respectively, throughout our pressure range. We note that at 1 atm, melittin is either fully associated or dissociated under those conditions, irrespective of protein concentration (Talbot et al., 1979). At these salt concentrations, we see no effect of protein concentration on the apparent degree of association, as indicated by the emission spectra or anisotropy measurements. Therefore, we took the values at the extremes of salt concentration to be indicative of the behavior of the monomer and tetramer.

The spectra of monomeric and tetrameric melittin do display small red shifts with increased pressure, even though no change in association is taking place (Figure 3). The small red shift of the monomer, which evidently exists nearly as a random coil at 1 atm (Lauterwein et al., 1980; Jentsch, 1969), and the somewhat larger shift of the tetramer (Figure 3) are a little bigger than those seen previously under pressure for indole derivatives in solution (Politis & Drickamer, 1981) and tryptophan in solution or in proteins (Li et al., 1976). Our results can best be explained by Weber's suggestion that increased pressures result in increased penetration of water to the interior of the protein (Li et al., 1976). This explanation is also supported by the slight decline in lifetime with pressure of the tetrameric form. However, the degree of solvent relaxation is likely to affect these spectral shifts, and the effect of pressure on this process is virtually unknown (MacGregor, 1983). We note that the reversibility of the spectral and anisotropy changes upon decompression is a good indication that the structural perturbations visited upon melittin by pressure are also reversible and that we are dealing with a true equilibrium.

The degree of dissociation, α (1 = complete dissociation), was calculated from the anisotropy data by using eq 4 and smoothed data sets for pressure-dependent anisotropies of the monomer and tetramer. A plot of ln $[\alpha^4/(1-\alpha)]$ vs. pressure is shown in Figure 4. A tetramer dissociation whose ΔV is pressure independent would appear in Figure 4 as a straight line with a slope equal to ΔV (eq 5). For melittin, ΔV is clearly dependent upon pressure. Initially, ΔV is large but drops off nearly to zero at pressures near 1.5 kbar (Figure 5). Also, within our experimental error, the curves in Figure 4 for different protein concentrations in the same salt solution are essentially identical and parallel. We note that the greater apparent noise at $\alpha > 0.8$ and $\alpha < 0.2$ is a natural consequence of the difficulty of measuring α near its extremes. As is well-known (Weber, 1975), the ratio of protein concentrations encompassed in going from a 10% dissociated dimer to a 90% dissociated dimer is 630-fold; for the more cooperative tetramer formation, the ratio is only 54-fold. At 1 atm, melittin displays a protein concentration dependence of its association more comparable with this latter value (Talbot et al., 1979; Quay & Condie, 1983). The approximate parallelism of the lines in Figure 5 suggests that it does as well at high pressure. We thus are confident that melittin is not associating to form some higher (or lesser) aggregate under pressure. Finally, we note that α determined from measurements on the *N*-methyl-

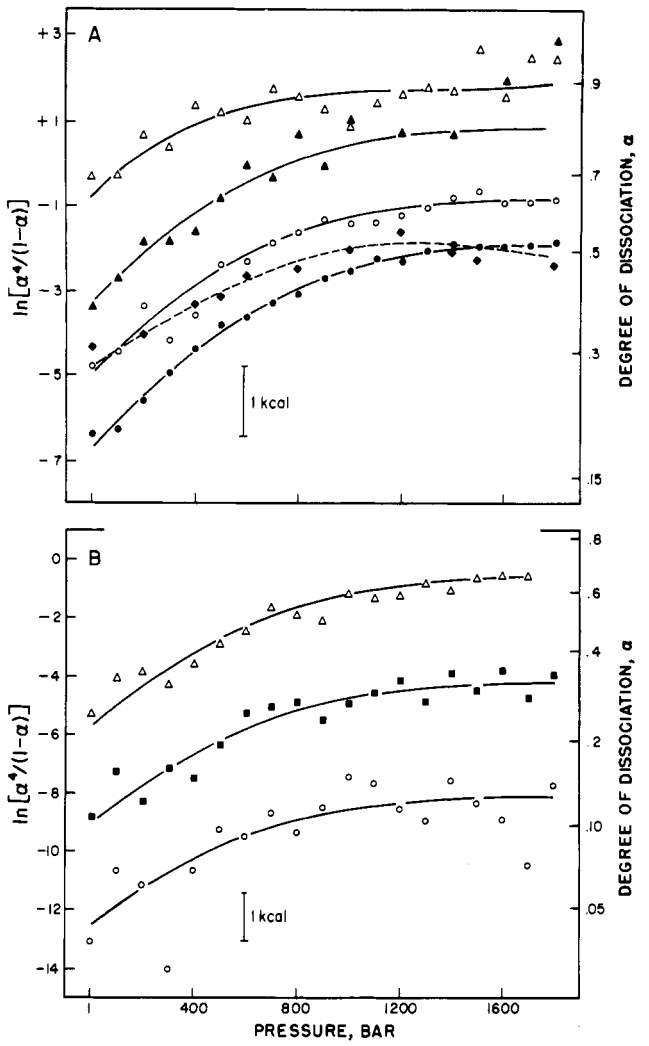

FIGURE 4: The degree of dissociation, α, and ln $[\alpha^4/(1-\alpha)]$ vs. pressure for melittin. These were calculated from eq 4 and 5 and the data in Figure 1. The vertical bars indicate the interval on the ln $[\alpha^4/(1-\alpha)]$ axis that corresponds to a change in ΔG of 1 kcal/mol. (A) 0.5 *m* NaCl; [melittin] = 10 (△), 50 (▲), 100 (○), and 200 (●) μM. (♦) Data for *N*-methylanthraniloylmelittin, measured as described under Materials and Methods. (B) 1.0 *m* NaCl; [melittin] = 10 (△), 50 (■), and 200 (○) μM.

anthraniloyl-labeled melittin displays a similar pressure dependence when compared with values obtained for unlabeled melittin.

The ΔV values of the dissociation reaction at different salt concentrations calculated from the slopes of the lines in Figure 4 (eq 5) are plotted vs. pressure in Figure 5. Negative values of ΔV for dissociation of protein oligomers are expected (Paladini & Weber, 1981a; Weber & Drickamer, 1983) and have generally been observed, even in ribosomes (Heremans, 1982). Our results show a dramatic pressure dependence, increasing from −187 mL/mol at 1 atm to less than −20 mL/mol at 1.8 kbar. Such a pressure dependence of ΔV has evidently been observed for glutamate dehydrogenase dissociation [unpublished results cited in Jaenicke (1981)], but by contrast, the ΔV of enolase changes from −30 mL/mol at 1 bar to −60 mL/mol at 1.5 kbar. The pressure dependence of ΔV measured for the labeled melittin is also in overt agreement with that observed for the unlabeled protein, confirming the results using the intrinsic tryptophan fluorescence.

The ΔV for dissociation of melittin at 1 atm is relatively large in comparison to those of other proteins. For instance, enolase exhibits a $\Delta V = -60$ mL/mol with a molecular weight of 90 000, and lactic dehydrogenase shows a volume decrease of 500 mL/mol for a molecular weight of approximately 144 000 (Schade et al., 1980). Melittin has a ΔV of −187 mL/mol. When the known molecular mass of 2846 g/mol and the density of 1.41 g/mL (Terwilliger & Eisenberg, 1982) are used, the volumes of the monomer and tetramer are 2018 and 8072 mL/mol, respectively. It thus exhibits a substantial decrease in total volume upon dissociation. It is important to note that melittin may differ from the enzymes cited above in that it unfolds almost completely upon dissociation (Lauterwein et al., 1980; Jentsch, 1969). Moreover, if the main contribution to ΔV comes from unfolding, it is unclear why it changes so much with pressure since the volume of the random-coil monomer is unlikely to be itself very pressure dependent, and proteins in general have shown themselves to be relatively incompressible (Gavish et al., 1983).

Finally, we also measured ΔV as a function of temperature as well as pressure for 160 μM melittin in 0.5 *m* NaCl; these results are presented in Figure 6. The anisotropies of monomer and tetramer used to calculate α (eq 4) were measured at each temperature and pressure as described under Theory. Also, we assumed that the pressure dependence of the relative quantum yield did not change dramatically with temperature. These results were used to examine the effect of pressure on the entropy change for the reaction, by using eq 7:

$$\left(\frac{\partial \Delta V}{\partial T}\right)_P = -\left(\frac{\partial \Delta S}{\partial P}\right)_T \tag{7}$$

We found that our determinations of ΔV were insufficiently accurate to judge whether a significant change in ΔS had occurred. These results are discussed below.

Discussion

We have shown that pressure dissociates melittin and that the efficacy of pressure in dissociating the protein decreases as the pressure is raised. Such behavior is somewhat novel in comparison to other proteins, but as stated in the introduction, melittin has some novel properties. In particular, tetrameric melittin is overtly similar to a detergent micelle. Its structure is configured to orient the hydrophobic amino acid side chains toward the center of the tetramer and to place the positively charged side chains a maximum distance from one another (Terwilliger & Eisenberg, 1982; Terwilliger et al., 1982). Moreover, the salt dependence of the melittin aggregation is the same as that found for micellization of detergents with a net charge; it is the reverse of that seen for enolase. Therefore, it is encouraging to note that pressure promotes micelle dissociation and that the ΔV for this process becomes less negative with pressure, becoming positive in some cases (Kaneshina et al., 1974; Offen, 1980; Hamann, 1962; Brun et al., 1978).

As mentioned in the introduction, melittin is held together by two types of interactions: hydrogen bonding within the subunits and "hydrophobic" bonding between the subunits. It thus provides a rare opportunity to study the behavior of these two important types of interactions in proteins under pressure. Of necessity, the pressure-induced dissociation must be explainable in terms of the pressure effects on these interactions.

How, then, do we account for the relatively large volume decrease upon dissociation, which shrinks so dramatically with pressure? The change in the volume difference between reactant (tetramer) and products (monomer) with pressure evidently requires that the tetramer shrink relative to the (random coil) monomer. It is unlikely that the monomer in its random coil can adopt a conformation with less volume or, particularly, less free volume. This is borne out by Zamyatnin's results (1972), and we also see no evidence in the

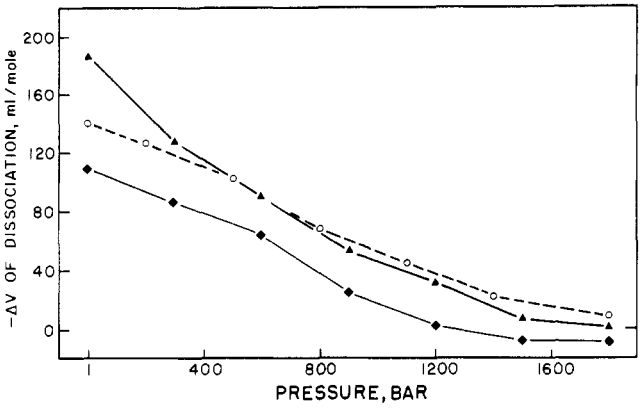

FIGURE 5: ΔV as a function of pressure for melittin dissociation. Data are shown for 200 μM melittin in 0.5 m NaCl (▲), 10 μM melittin in 1.0 m NaCl (○), and *N*-methylanthraniloylmelittin in 0.5 m NaCl (◆).

fluorescence spectra or anisotropy of the monomer to suggest a conformational change. One may also expect the tetramer to be relatively incompressible. Proteins in general are much less compressible even than water (Gavish et al., 1983). However, the anisotropies of the tetramer increase with pressure (Figure 1), indicating restricted motion of the tryptophan residues. The hydrophobic interior is the least dense portion of the tetramer (Terwilliger & Eisenberg, 1982), and aliphatic hydrocarbons (there are no aromatic residues apart from tryptophan) are the most compressible of liquids. Hence, it seems possible that the tetramer is relatively compressible, and as a result, the volume difference between the monomer and tetramer is decreased as the pressure is increased. The interiors of micelles are also compressible (Tanaka et al., 1974), and similar reasoning has been used to explain the pressure-induced dissociation of micelles. Compression of the tetramer would evidently require some distortion of the α helices. We note that α helices themselves seem to be rather stable under pressure; metmyoglobin requires approximately 6 kbar to begin denaturing at 20 °C and pH 6 (Zipp & Kauzmann, 1973).

In our opinion, compression of tetrameric melittin is the most probable cause of the large change in ΔV with pressure. However, there is an alternative explanation for the observed change in ΔV. We [along with others (Offen, 1980)] have implied that ΔV is the difference in partial molal volumes between melittin in the monomer and tetramer forms. This is not precisely correct. It is important to remember that we are pressurizing the whole system, including the solvent (Hvidt, 1975). In particular, the entire system (solvent and solutes) will configure itself to minimize its volume, consistent with the strength of its covalent and noncovalent interactions. Melittin behaves in many ways like a detergent micelle. It is relatively well understood that the principal forces which determine micelle formation are the repulsion of the charged head groups and, more important, hydrophobic "bonding". The source of the latter interaction is widely accepted to be primarily entropic in nature. More precisely, the entropy of the water (and thus the whole system) can be much greater with hydrophobic moieties being associated since it is not obliged to form clathrate-like cages around the solute (Tanford, 1980). We feel that a similar effect may contribute to the volume changes we observed and those observed for micelle formation. In particular, one of the principal effects of pressure in this range could be alteration of the microstructure of the water and thus the entropy gained by isolating the hydrophobic side chains of melittin from the aqueous phase. Kaneshina et al. (1974) found that such an effect was indeed responsible, in part, for their observations of sodium dodecyl sulfate micellization. More precisely, they measured ΔV for the micellization reaction as a function of temperature and pressure. By employing eq 7, they found that ΔS for the reaction also changed with pressure, thus suggesting that pressure-induced changes in water structure were responsible. As might be expected, most of the free energy of melittin self-association is entropy derived (Quay & Condie, 1983), and thus one might also suspect that pressure promotes dissociation of melittin for the same reason.

We therefore measured ΔV for the reaction as a function of pressure at five temperatures from 5 to 45 °C. The results of a typical set of experiments are indicated in Figure 6. We note that our experimental error in these measurements is rather large, particularly below 400 bar; this is due to the lower density of data points and the likelihood that the pressure dependences of the relative quantum yield, pH, emission spectrum, and salt solution viscosity may vary somewhat with temperature. Evidently a very large array of experiments would be necessary to isolate the effects of all these parameters on such measurements. Nonetheless, our limited experiments did not reveal any systematic variation of ΔV with temperature, irrespective of pressure. Therefore, we feel that ΔS is largely pressure independent and that the observed change in ΔV with pressure is mainly due to compression of the tetramer.

It is important to note, however, that if the total change in the free energy of association is solely derived from an entropy change, the resulting temperature dependence of ΔV will be barely detectable by our method. For instance, at 25 °C in the interval 1–100 bar, ΔG for the reaction decreases by about 0.42 kcal/mol (Figure 4). If we assume that this entire change is due to a change in entropy [e.g., $\Delta(\Delta G) = -T\Delta(\Delta S)$], then the corresponding entropy change would be 1.41 cal K^{-1} mol^{-1}. Expressing the interval of pressure in convenient units of calories per milliliter (1 cal/mL = 41.8 bar), we can calculate the expected change in ΔV over a 40 °C range in temperature as follows:

$$-\left[\frac{\Delta(\Delta S)}{\Delta P}\right]_T = \frac{1.47 \text{ cal K}^{-1} \text{ mol}^{-1}}{2.39 \text{ cal/mL}} = \left[\frac{\Delta(\Delta V)}{\Delta T}\right]_P = \frac{\Delta(\Delta V)}{40 \text{ K}} \quad (8)$$

Using eq 7 yields $\Delta(\Delta V)$ = 23.5 mL/mol. Thus, we would expect that ΔV for the reaction (near 1 atm) would change *at most* by 24 mL/mol going from 5 to 45 K, if the pressure acted solely to change the entropy of the reaction. Such a change is indicated in Figure 6 (---). Clearly, our data are not accurate enough to judge the relative importance of entropy changes in determining the pressure-induced dissociation of melittin, but evidently they could be substantial.

In conclusion, then, we have shown that pressure dissociates tetrameric melittin with decreasing efficacy as the pressure is raised. It is interesting to notice that increased pressures caused the dissociation of both enolase (Paladini & Weber, 1981a) and melittin, even though the forces stabilizing aggregation are likely to be mixed in the case of enolase but are hydrophobic in the case of melittin. Such similar effects probably reflect the generality of Weber's suggestion that subunit association results in trapped volume at the subunit interface. However, our anisotropy data, which suggest that the tetramer is compressible, are not in agreement with Weber's prediction that the subunit interface of proteins is not compressible. Also, our conclusions are not in agreement with the adiabatic data (Gavish et al., 1983). However, the melittin tetramer interface is clearly a region of low density and weak

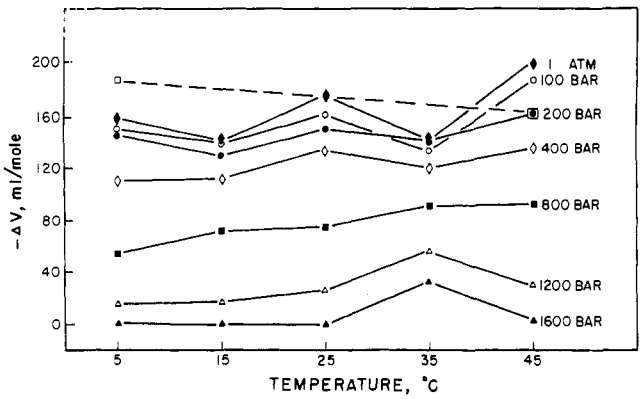

FIGURE 6: ΔV as a function of temperature and pressure for 150 μM melittin in 0.5 m NaCl. The dashed line indicates the expected change in ΔV with temperature if the pressure-induced change in melittin association is entirely due to entropy change.

intermolecular interaction (Terwilliger & Eisenberg, 1982; Terwilliger et al., 1982) and consequently may be more compressible than most proteins, such as those studied by Gavish et al. (1983). The other hypothesis that could explain the decreased ΔV with pressure is based on the work of Kaneshina et al. (1974). They found that pressure-induced changes in the entropy change for the micellization of sodium dodecyl sulfate (SDS) were in part responsible for behavior similar to that observed for melittin. We attempted similar measurements, but our data were too noisy to accurately judge the influence pressure might have on the entropy, though it might be significant. It is certainly clear from our results, however, that pressure in this range can have a substantial effect on the integrity of biomolecules that are principally stabilized by hydrophobic interactions.

Acknowledgments

We are grateful to Drs. E. Bucci and P. M. Torgerson for critical reading of the manuscript and to Dr. H. L. T. Mobley for electrophoresis of the melittin sample. We are especially grateful to Dr. A. Hermetter for the gift of the *N*-methylanthraniloylmelittin.

Registry No. Melittin, 37231-28-0.

References

Brandts, J. F., Oliveira, R. J., & Westort, C. (1970) *Biochemistry 9*, 1038–1047.

Bridgman, P. W. (1926) *Proc. Am. Acad. Arts Sci. 61*, 57–99.

Brun, T. S., Hoiland, H., & Vikingstad, E. (1978) *J. Colloid Interface Sci. 63*, 89–96.

Chryssomallis, G. S., Torgerson, P. M., Drickamer, H. G., & Weber, G. (1981) *Biochemistry 20*, 3955–3959.

Dufourcq, J., & Faucon, J.-F. (1977) *Biochim. Biophys. Acta 467*, 1–11.

Faucon, J. F., Dufourcq, J., & Lussan, C. (1979) *FEBS Lett. 102*, 187–190.

Gavish, B., Gratton, E., & Hardy, C. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 750–754.

Georghiou, S., Thompson, M., & Mukhopadhyay, A. K. (1981) *Biochim. Biophys. Acta 642*, 429–432.

Habermann, E. (1972) *Science (Washington, D.C.) 177*, 314–322.

Habermann, E. (1980) in *Natural Toxins* (Eaner, D., & Wadstrom, T., Eds.) pp 173–181, Pergamon Press, Elmsford, NY.

Habermann, V. E., & Jentsch, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 37–50.

Hamann, S. D. (1962) *J. Phys. Chem. 66*, 1359–1361.

Harrington, W. F., & Kegeles, G. (1973) *Methods Enzymol. 27D*, 306–345.

Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.

Hook, J. W., & Drickamer, H. G. (1978) *J. Chem. Phys. 69*, 811–815.

Horne, R. A., & Johnson, D. S. (1966) *J. Phys. Chem. 70*, 2182–2190.

Hvidt, A. (1975) *J. Theor. Biol. 50*, 245–252.

Jaenicke, R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 1–67.

Jentsch, J. (1969) *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 24B*, 33–35.

Kaneshina, S., Tanaka, M., Tomida, T., & Matuura, R. (1974) *J. Colloid Interface Sci. 48*, 450–460.

Lakowicz, J. R., & Thompson, R. B. (1983) *Biochim. Biophys. Acta 732*, 359–371.

Lakowicz, J. R., Maliwal, B. P., Cherek, H., & Balter, A. (1983) *Biochemistry 22*, 1741–1752.

Lauterwein, J., Brown, L. R., & Wuthrich, K. (1980) *Biochim. Biophys. Acta 622*, 219–230.

Li, T. M., Hook, J. W., Drickamer, H. G., & Weber, G. (1976) *Biochemistry 15*, 3205–3211.

MacGregor, R. B. (1983) Ph.D. Thesis, University of Illinois at Urbana–Champaign.

Mollay, C., & Kriel, G. (1973) *Biochim. Biophys. Acta 316*, 196–203.

Neuman, R. C., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687–2691.

Offen, H. W. (1980) *Rev. Phys. Chem. Jpn. 50*, 97–118.

Paladini, A. A., & Weber, G. (1981a) *Biochemistry 20*, 2587–2593.

Paladini, A. A., & Weber, G. (1981b) *Rev. Sci. Instrum. 52*, 419–427.

Politis, T. G., & Drickamer, H. G. (1981) *J. Chem. Phys. 75*, 3203–3210.

Quay, S. C., & Condie, C. C. (1983) *Biochemistry 22*, 695–700.

Schade, B. C., Rudolph, R., Ludemann, H.-D., & Jaenicke, R. (1980) *Biochemistry 19*, 1121–1126.

Sergeevich, V. I. Zhuze, T. P., & Chestnov, A. I. (1953) *Izv. Akad. Nauk SSSR, Otd. Tekh. Nauk*, 896–903.

Sessa, G., Freer, J. H., Calacicco, G., & Weissman, G. (1969) *J. Biol. Chem. 244*, 3575–3582.

Suzuki, K. (1972) *Methods Enzymol. 26*, 424–452.

Talbot, J. C., Dufourcq, J., deBony, J., Faucon, J. F., & Lussan, C. (1979) *FEBS Lett. 102*, 191–193.

Tanaka, M., Kaneshina, S., Shin-no, K., Ikajima, T., & Tomida, T. (1974) *J. Colloid Interface Sci. 46*, 132–138.

Tanford, C. (1980) *The Hydrophobic Effect*, 2nd ed., Wiley-Interscience, New York.

Terwilliger, T. C., & Eisenberg, D. (1982) *J. Biol. Chem. 257*, 6016–6022.

Terwilliger, T. C., Weissman, L., & Eisenberg, D. (1982) *Biophys. J. 37*, 353–361.

Trudell, J. R., Hubbell, W. L., Cohen, E. N., & Kendig, J. J. (1973) *Anesthesiology 38*, 207–211.

Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 10*, 363–374.

Weber, G. (1975) *Adv. Protein Chem. 29*, 1–85.

Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89–112.

Zamyatnin, A. A. (1972) *Prog. Biophys. Mol. Biol. 24*, 109–123.

Zipp, A., & Kauzmann, W. (1973) *Biochemistry 12*, 4217–4228.