Conformation and Aggregation of Melittin: Dependence on pH and Concentration†

Jake Bello,* Helene R. Bello, and Edward Granados

ABSTRACT: Melittin, a 26-residue peptide from bee venom, is transformed from a largely random to a largely α-helical conformation at elevated pH. At 3×10^{-5} M melittin, circular dichroism spectra show a transition with a pK near 9.6. At 8×10^{-5} M, two approximately equal transitions occur with pKs at 7.2 and 9.6. At 6×10^{-4} M, a single transition is seen with a pK of 6.8, followed by a more gradual increase to at least pH 11. The transitions near pH 7 presumably arise from deprotonation of the α-amino group. When the amino groups are acetylated or succinylated, a 60% α-helical conformation is adopted at neutral or low pH. The acylated melittins form more stable oligomers than does native melittin.

Melittin, the major component of the venom of the bee *Apis mellifera*, is of interest because it lyses cell membrane and exhibits a variety of other biological effects (Habermann, 1972; Schroeder et al., 1971). Melittin has 26 residues of which 6 are positively charged, the α-amino of the terminal glycine, 3 ϵ-amino groups of lysines at positons 7, 21, and 23, and 2 guanidino groups of arginine at positions 22 and 24. There are no negative charges. The amino acid sequence is H_2N-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2. The sequence is amphiphilic, since its C-terminal third is hydrophilic, while the remainder has a high proportion of apolar residues. Aggregation of melittin to a tetramer is promoted by high salt, high melittin concentration, and high pH. Aggregated melittin has been likened to a lipid aggregate and is of interest as a model for protein folding and as a model for the N-terminal region of nascent preproteins (Knöppel et al., 1979).

Talbot et al. (1979) showed by ORD[1] that at pH 7.5 the helical content of melittin at 1.8×10^{-4} M rises from 12% in nearly zero salt to 65% above 1 M NaCl. Dawson et al. (1978) found a very low helix content for melittin in 0.15 M NaCl at pH 7.4. Knöppel et al. (1979) reported that at pH 8.8, at a concentration of 1.07×10^{-4} M and an ionic strength of 0.05 M, the helical content calculated from CD is 48%. Lauterwein et al. (1980) found that between 5.8×10^{-6} and 6.7×10^{-4} M melittin in 0.05 M phosphate, pH 7, $[\theta]_{222}$ changed from -4000 to $-16\,000$ deg cm^2 dmol^{-1}. Faucon et al. (1979) and Talbot et al. (1979) found that aggregation is promoted by NaCl and by elevated melittin concentration. Brown et al. (1980) studied the ^{1}H NMR spectrum and ultracentrifugation of melittin and found aggregation to tetramer at elevated concentration at neutral pH, at low pH in 1.5 M NaCl, at high concentration in low pH, salt-free solution, and at pH 9 in salt-free solution. They found that deprotonation of the α-amino enhances the tendency to aggregate.

We present circular dichroism data for melittin showing that the conformation is a complex function of concentration, salt, and pH and that at least three conformational states can exist at neutral pH.

Experimental Procedures

Materials. Melittin was obtained from Sigma Chemical Co. and was homogeneous on gel filtration through Sephadex G-50. It has been reported that up to 20% of melittin has its terminal NH_2 formylated (Habermann & Jentsch, 1967; Lübke et al., 1971). We used the melittin without separation of any formylated component. Acetylated melittin was prepared by reaction with acetic anhydride using 6 μL of anhydride to 2 mg of melittin in 2 mL of 0.02 M phosphate buffer, pH 7.2. After acetylation, the pH was readjusted to 7.2 with NaOH. Succinylation was done in the same way by using 6 mg of anhydride. Reaction of melittin with 2,4,6-trinitrobenzenesulfonic acid, 2-chloro-3,5-dinitrobenzoic acid, fluorescamine, and 1-chloro-4-nitrobenzofurazan was carried out at an equimolar ratio at 26 °C, pH 8.2; the rate of reaction with the last reagent was followed at 350 nm with a Cary 219 spectrophotometer.

Optical Measurements. The concentration of melittin was calculated from the absorbance at 280 nm, using 5600 cm^{-1} M^{-1} for the sole chromophore, the tryptophyl residue. CD spectra were measured with a Jasco CD-5 apparatus; absorbance of solutions was less than 0.5. Fluorescence polarization was measured with an Aminco-Bowman spectrophotofluorometer fitted with an Applied Physics Co. Model 1108 photon counter. Excitation was at 280 nm, and emission was measured at 420 nm, with the excitation beam filtered with a Corning 7-54 filter. The excitation beam was not polarized. Polarization was calculated from

$$P = \frac{I_v - gI_H}{I_v + gI_H}$$

where I_v and I_H are the intensities with vertical and horizontal emission-side polarizer, respectively, and g is a correction factor obtained with fluorescein which shows complete depolarization.

Molecular Weights by Gel Filtration. Gel filtration of mellitin, acetylated melittin, and succinylated melittin was done in a 0.9 × 47 cm column of Sephadex G-50 eluted by 0.02 M sodium phosphate, at an elution rate of 9 mL/h. The calibration standards were ribonuclease S peptide, insulin B chain, ribonuclease S protein, and myoglobin. S peptide and S protein were obtained from Sigma Chemical Co., and insulin B chain was from Boehringer Mannheim. Gel filtration of acetylated and succinylated melittin was done on the acylation

† From the Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York 14263. *Received December 1, 1981; revised manuscript received August 12, 1981.* Supported in part by Grant GM 24525 from the Institute of General Medical Sciences, National Institutes of Health, and Grant PCM-7903666 from the National Science Foundation.

[1] Abbreviations: ORD, optical rotatory dispersion; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-0461$01.25/0

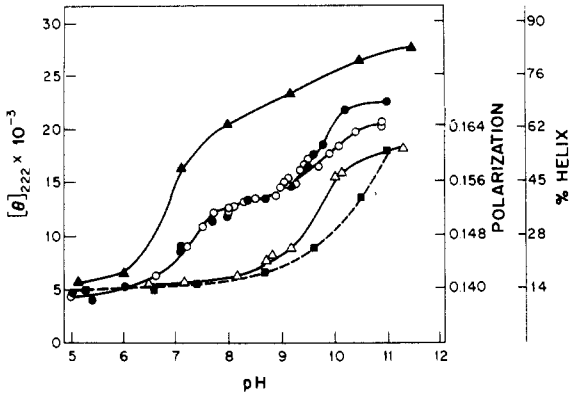

FIGURE 1: Ellipticity, % helix, and polarization of fluorescence of melittin as functions of pH. (■) Polarization, 3×10^{-5} M in 0.02 M sodium phosphate. All other curves are for $[\theta]_{222}$ and % helix as follows: (△) 3×10^{-5} M melittin in 0.02 M sodium phosphate; (○) 8×10^{-5} M in 0.02 M sodium phosphate; (●) 8×10^{-5} M in phosphate–glycine–NaCl; (▲) 6.3×10^{-4} M in phosphate–glycine–NaCl.

reaction mixtures, after ample time for complete hydrolysis of the excess anhydride; therefore gel filtration of control melittin was done on solutions containing the same concentration of acetate or succinate ion, 6×10^{-3} and 3×10^{-3} M, respectively. A control melittin without acetate or succinate gave the same result. Fractions of 22 drops were collected in graduated tubes corresponding to 1.8 mL for buffer. At the peptide elution regions, the volume per tube was less because of surface tension changes. Fractions were read with a Cary 219 spectrophotometer at 280 nm, except ribonuclease S peptide and succinylated melittin at the lowest concentration used, which were read at 220 nm. In most cases, 0.25 mL of 2–3 mg/mL concentration was applied to the column. Some runs were also done with melittin at 10 and 40 mg/mL and with succinylated melittin at initial concentrations as low as 0.17 mg/mL.

Results

The $[\theta]$ values are based on concentrations derived from absorption spectra and an ϵ_{max} value of 5600 M^{-1} cm^{-1}. The absorption spectrum was recorded for each CD spectrum to account for pipetting uncertainties and for volume changes accompanying pH adjustment. There remains the possibility of error arising from a possible change in ϵ_{max} accompanying aggregation and transconformation. This should not markedly affect the main features of the data. The CD titration curve of melittin is strongly dependent on the concentration of the peptide, as shown in Figure 1. At the lowest concentration studied, about 3×10^{-5} M (0.084 mg/mL), there is essentially no change in $[\theta]_{222}$ up to about pH 8, after which the major change in $[\theta]$ sets in leading to a value at pH 11 of about -18×10^3 deg cm^2 $dmol^{-1}$ for $[\theta]_{222}$, equivalent to about 51% helix, based on -35×10^3 for 100% helix. The midpoint is at pH 9.6 which is in the range of the pK of typical ϵ-amino groups, but perhaps a bit low. Two of the three lysyl residues are in the sequence Lys-Arg-Lys-Arg with a high density of positive charge, which could lower the pKs.

On increasing the melittin concentration 2.7-fold to about 8×10^{-5} M, a marked change appears in the CD titration curve. Figure 2 shows some representative CD spectra at this concentration. There are now two distinct steps in the curve (Figure 1), the first with a pK near 7.2 and the second with a pK near 9.6. There is some dependence on ionic strength, with the second step for 0.12 M salt being shifted to somewhat lower pH than that at 0.02 M. This is the opposite of expectation since higher ionic strength usually shifts upward the pK of a multiple-cationic polymer.

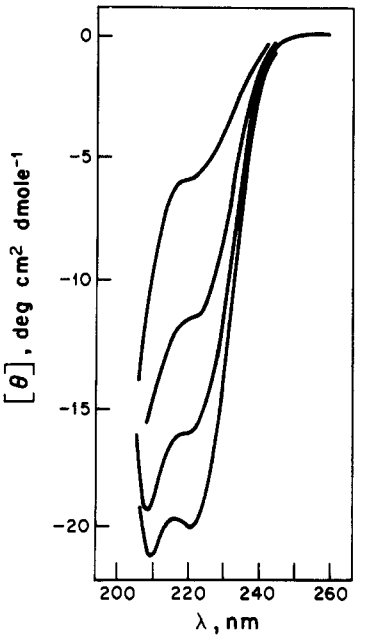

FIGURE 2: CD spectra of melittin in 0.02 M sodium phosphate at (from top to bottom) pH 4.8, 7.27, 8.7, and 10.5.

On increasing the melittin concentration again, by a factor of about 8, to 6.3×10^{-4} M, another marked change in the CD titration curve occurs. There is a large increase in the magnitude of $[\theta]_{222}$ between pH 6 and 8, changing from about -6×10^3 to -19×10^3 deg cm^2 $dmol^{-1}$. There appears to be a pK near 6.8, distinctly below that at 8×10^{-5} M. From pH 8 to 11.5 there is a gradual increase to -27.5×10^3 deg cm^2 $dmol^{-1}$, a considerably larger $[\theta]_{222}$ than at the lower concentrations. At the highest pH studied, the α-helix content appears to be about 80%. This is the largest $[\theta]_{222}$ and apparent helicity yet reported for melittin. At pH 11.5 some turbidity was noted, which might decrease the ellipticity below its intrinsic value.

The increase in the magnitude of $[\theta]_{222}$ at pH 7.2 as the concentration of melittin is increased is in accord with the optical rotatory data of Talbot et al. (1979) for melittin at pH 7.5. Effects of high ionic strength observed by others were confirmed by results with melittin in 1 M KCl at pH 8.2 at 3×10^{-5} and 8×10^{-5} M melittin, for which $[\theta]_{222}$ was -19.4×10^3 and -21.7×10^3, respectively. The latter value rose 5% after 24 h. Heating melittin in 1 M KCl to 61 °C caused precipitation. Our results at 8×10^{-5} and 6.3×10^{-4} M melittin at pH 7.4 are markedly different from that of Dawson et al. (1978), who found essentially no helix for melittin at 3.5×10^{-4} M, pH 7.4, and 0.15 M NaCl. The reason for this discrepancy is not known, but the result of Dawson et al. is at odds with all other reported results. We also have found a result different from that of Dawson et al. for melittin in 70% ethanol. We found a distinctly α-helical CD spectrum with $[\theta]_{222} = -25 \times 10^3$, with 0.1, 0.25, and 1 mg/mL melittin (3.5×10^{-5}–3.5×10^{-4} M) with or without 0.02 M phosphate buffer. Dawson et al. found (with 1 mg/mL and apparently no buffer) a quite different spectrum, neither α helical, β structure, nor random (see their Figure 5).

Depolarization of Fluorescence. We measured the depolarization of fluorescence of the tryptophan residue of melittin. At 3×10^{-5} M the polarization–pH curve and the CD–pH curve are parallel up to about pH 8.5 but diverge at higher pH, with polarization continuing to rise after $[\theta]$ has leveled

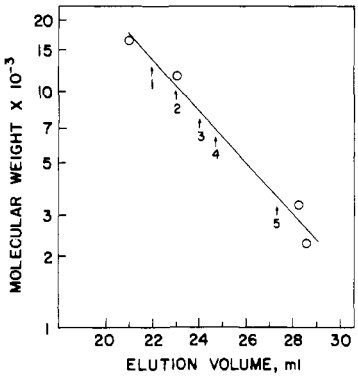

FIGURE 3: Molecular weights by gel filtration. Circles are, from highest to lowest molecular weight, myoglobin, RNase S protein, insulin B chain, and RNase S peptide. The arrows are the following: (1) succinylated melittin, 2 mg/mL; (2) melittin, 40 mg/mL; (3) melittin, 10 mg/mL; (4) acetylated melittin, 2 mg/mL; (5) melittin, 3 mg/mL.

off (Figure 1). We were not able to obtain useful data at 8 $\times 10^{-5}$ M, because the values at various pH values gave a zigzag curve, which was irreproducible from run to run and even for the same solution at different times. But the reproducibility of the CD spectra at 8 $\times 10^{-5}$ M is good, as is shown by the adherence of the large number of experimental points to the curve (Figure 1). This curve shows all of the data points obtained; none were discarded.

CD of Acetylated and Succinylated Melittin. The CD effects resulting from pH changes arise from the titration of the amino groups since these are the only groups titratable below the range of the guanidinium groups. At the highest pH values studied, some effect of titration of guanidinium groups may begin to enter. Since the CD of melittin depends on the state of protonation of the amino groups, we measured the CD spectra of acetylated and succinylated melittin. These are also of interest from the work of Habermann & Kowallek (1970), who found that these showed reduced ability to lyse erythrocytes and that succinylated melittin showed much reduced ability to lower the surface tension of water.

Acetylated melittin at 3 $\times 10^{-5}$ and 8 $\times 10^{-5}$ M at pH 7.2 shows a high degree of helicity, with $[\theta]_{222}$ equal to -20×10^3 and -22×10^3, respectively, corresponding to about 60% helix. At 61 °C, the helicity of 8 $\times 10^{-5}$ M acetylated melittin fell by 60%. At pH 11.2, there is no increase in helicity, indicating that all of the amino groups (or all of them that matter for conformation) are acetylated. This is supported by the fact that acetylation with a 3-fold amount of acetic anhydride gave the same results. In 0.75 M KCl, pH 7.2, $[\theta]_{222}$ of 8 $\times 10^{-5}$ M acetylated melittin is -20×10^{-3} or 57% helix; on heating, precipitation occurred. Succinylated melittin at 8 $\times 10^{-5}$ M and at 3 $\times 10^{-5}$ M had a helix content at pH 7.2 (net charge -2) similar to that of acetylated melittin, $[\theta]_{222}$ of -22×10^3 and -21×10^3, about 63% helix. At pH 2.9 (net charge +2, the same as for acetylated mellitin), the helicity was 50% for succinylated mellitin, significantly less than the 63% at pH 7.2. At pH 2.9 and 7.2, 1 M KCl caused precipitation of succinylated melittin at room temperature.

On lowering of the concentration of acetylated melittin (pH 7.2) to 3 $\times 10^{-6}$ M, $[\theta]_{222}$ fell to -7×10^3, or about 20% helix. But for succinylated melittin (pH 7.2) at 3 $\times 10^{-6}$ M, $[\theta]_{222}$ fell only slightly to -19.5×10^3.

Molecular Weights. The apparent molecular weights of melittin and of acetylated and succinylated melittin were measured by gel filtration on Sephadex G-50 with elution by 0.02 M sodium phosphate, pH 7.2 (Figure 3). The molecular weight of melittin itself depended on the concentration at which it was eluted. When the concentration in the peak tube was about 3 $\times 10^{-5}$ M (corresponding to the lowest curve of Figure 1), the molecular weight was 3.6 $\times 10^3$, instead of 2.8 $\times 10^3$ required by the molecular formula, but in agreement with the value of 3.5 $\times 10^3$ found by Talbot et al. (1979). When a more concentrated melittin solution (10 mg/mL) was applied to the column so that the eluted peak concentration was 9 $\times 10^{-5}$ M (near that of the middle curve of Figure 1), the molecular weight was 7.6 $\times 10^3$. When a 40 mg/mL solution was applied, resulting in a peak concentration of 2.4 $\times 10^{-4}$ M, the apparent molecular weight was 1 $\times 10^4$. Thus, melittin under the conditions of the first step in the middle CD titration curve of Figure 1 may be dimeric. Molecular weight by gel filtration is dependent on molecular shape and density (Ackers, 1975). Also since melittin is an associating system, a more detailed analysis will be required. Lübke et al. (1971) found melittin to be dimeric with a molecular weight of 6 $\times 10^3$ at pH 5.5, at an unspecified concentration. Knöppel et al. (1979) found that melittin applied at 3 mg/mL (1.1 $\times 10^{-3}$ M) is eluted from Sephadex G-50 by 0.1 M Tris-HCl, pH 8.8, at close to the position of lysozyme, corresponding to a molecular weight of about 14 $\times 10^3$, or a tetramer at least. The higher molecular weight found by Knöppel et al. probably arises from the higher ionic strength and pH in their eluting solution. The peak absorbance (their Figure 3) corresponded to 8.9 $\times 10^{-5}$ M melittin. Our Figure 1 shows melittin under approximately these conditions to have a $[\theta]_{222}$ of -15×10^3, corresponding to about 40% helix. The molecular weights of acetylated and succinylated melittin were found to be about 7 $\times 10^3$ and 13.5 $\times 10^3$, respectively. The peak concentration of acetylated melittin as eluted was about 3 $\times 10^{-5}$, as in the lowest concentration of native melittin used for CD (Figure 1). Thus, acylated melittins at pH 7.2 form aggregated helical structures more easily than does melittin. Gel filtration of succinylated melittin diluted to 0.2 mg/mL, so that it was eluted at a peak concentration of about 3 $\times 10^{-6}$ M, gave an elution volume identical with that at a peak concentration of 3 $\times 10^{-5}$ M. The marked decrease in helicity on dilution of acetylated melittin to 3 $\times 10^{-6}$ M, noted above, must be accompanied by disaggregation, although the molecular weight was not measured. Succinylated melittin is more stable in the aggregated form than is acetylated melittin.

Other Chemical Modification Studies. There appears to be a single critical amino group involved in the first increase in helicity with pH, at the middle and high concentrations, with an apparent pK of about 7; this group might be susceptible to selective modification. Reactions at pH 8.2 were carried out on 10^{-4} M melittin with a 1:1 molar ratio of several widely used agents, 2,4,6-trinitrobenzenesulfonic acid, 4-nitro-7-chlorobenzofurazan (NBD chloride), and fluorescamine. All gave insoluble derivatives. With the reagent 2-chloro-3,5-dinitrobenzoic acid (Bello et al., 1979), also at a 1:1 ratio, there was no reaction at pH 8.2. At pH 10.4, an insoluble product formed. The rate of the reaction with nitrochlorobenzofurazan was measured by the change in absorbance at 350 nm. The rate of change of absorbance (which includes turbidity) at pH 8.2 was 25 times as large as at pH 7.2. This is anomalous if the pK is really at 7.2, and more so if it is 7.8 as found by Brown et al. (1980) by NMR. However, we do not wish to belabor this point since the turbidity must introduce considerable uncertainty. Melittin has a large proportion of hydrophobic residues, yet it is very water soluble. It is interesting that a single aromatic substitution resulted in a marked decrease in solubility, even when a carboxylate is introduced with the aryl group. Thus, a small

modification, presumably at the terminal-amino group, appears to bring the very soluble melittin over the threshold to insolubility. However, acetylation or succinylation did not cause insolubilization, so that the loss of positive charge alone is not the cause.

Discussion

Helix formation and aggregation depend on pH, ionic strength, and melittin concentration. Confirming results of others, we find that high salt results in a larger degree of α helix. Most of our work has been at relatively low salt, 0.02 and 0.12 M. We attend particularly to the effect of deprotonating ammonium groups. At the lowest concentration studied, 3×10^{-5} M melittin, titration of the terminal ammonium group has no effect on conformation, while titration of one or more ϵ-ammonium groups results in formation of about 50% α helix. The results on depolarization of fluorescence at 3×10^{-5} M melittin are mainly of interest in confirming the absence of conformational change below pH 8 and in showing that aggregation does not occur in this pH range. At 8×10^{-5} M, deprotonation of the α-ammonium induces partial α-helical conformation, 35%. Deprotonation of one or more ϵ-ammonium groups results in additional helix, about 30%, or a total of about 65%. At the highest concentration, 6.3×10^{-4} M, titration of the α-ammonium results in about twice as much helix as at 8×10^{-5} M, with additional helix developing at higher pH. The scale of helicity in Figure 1 is based on $[\theta]_{222} = 35\,000$ for 100% helix. The use of $[\theta]_{208}$, where β and random structures contribute little to $[\theta]$, with the formula of Greenfield & Fasman (1969) gives substantially similar results. The highest apparent helicity observed is about 80%, or 21 residues out of 26. By the two-wavelength method of Goren et al. (1977), the α-helix content was within 10% of that calculated at 222 nm. Terwilliger et al. (1982) have elucidated the structure of crystalline melittin and found it completely α helical except for a bend at residues 10–11. Our estimate of a maximum helicity of 80% α helix or 21 residues is in close agreement. The slight turbidity noted at pH 11.5 for 6.3×10^{-4} M melittin maybe decreased the magnitude of $[\theta]_{222}$; otherwise the agreement might have been better. While our maximum helicity occurs at high pH, the crystals were prepared at neutral pH. The latter result again indicates the ability of high concentration to overcome barriers to structural change.

The apparent pK values are of interest. Lauterwein et al. (1980) found by NMR a pK of 7.8 for the α-amino group of melittin, a typical pK for a terminal glycine. Our pK of 7.2 for 8×10^{-5} M melittin is significantly lower, and a pK of 6.8 for 6×10^{-4} M melittin is still lower, even though the work of Lauterwein et al. was done with more concentrated melittin, 3×10^{-3} M. Brown et al. (1980) noted that the strong concentration dependence of aggregation is indicative of a cooperative effect. This is also supported by the shift in pK on going from 8×10^{-5} to 6.3×10^{-4} M melittin and by the downward shift of the second pK at 8×10^{-5} M melittin at higher salt, since salt promotes aggregation. The lowering of the pK between 8×10^{-5} and 6×10^{-4} M melittin accompanies a structural change.

The gel filtration data of Knöppel et al. (1979) under conditions corresponding to the plateau at $[\theta]_{222} = -15 \times 10^3$ for the middle curve of Figure 1 (except for the use of 0.1 M Tris-HCl instead of 0.1 M KCl plus 0.02 M phosphate) indicate a tetramer. Since melittin is tetrameric at higher concentration, where greater α-helical character is reached, these results indicate that at least two tetrameric structures are formed. The data of Figure 4 suggest that there may be six conformation–aggregation states of melittin in the pH and concentration ranges studied.

At 3×10^{-5} M melittin, deprotonation of the α-ammonium does not sufficiently reduce the net positive charge to permit aggregation and transconformation; at 8×10^{-5} M and above, this is permitted. Since melittin has a high charge density, it is reasonable that aggregation would be opposed by electrostatic repulsions. The aggregation induced by high salt is consistent with this. Another possible effect of high salt is the salting-out of melittin monomers to generate tetramer through hydrophobic bonding. The hydrophobic effect of 1 M KCl in promoting aggregation may be very crudely estimated from data on solubility of hydrocarbons in water and 1 M NaCl obtained by Morrison (1952) and Morrison & Billett (1952), from which Tanford (1980) has calculated the ΔG of transfer of hydrocarbon from water to 1 M salt and presented an equation connecting ΔG to the number of CH_3 and CH_2 groups. From the number of CH_3, CH_2, and CH groups in the hydrophobic side chains of melittin, including Trp, a ΔG of transfer of about 4 kcal/mol is obtained, corresponding to a factor of 800 in the solubility. If the hydrophobic side chains are tightly packed in the interface, an additional contribution to ΔG would result (Bello, 1977, 1978). Precipitation of melittin in KCl or at high pH by heat is in accord with hydrophobic aggregation. Morrison and Morrison and Billett found that the solubility of small aliphatic hydrocarbons in 1 M KCl decreases with increasing temperature, although less so in water.

In the experiments on modification by trinitrobenzenesulfonic acid, etc., the solubility of singly modified melittin became less than 10^{-4} M in 0.02 M phosphate. The solubility of melittin in 0.02 M phosphate is greater than 200 mg/mL or 668×10^{-4} M, the highest concentration we have made. Under the assumption that the activity coefficients are the same for melittin and modified melittins, the free energy difference is at least 4 kcal/mol, a value similar to that for precipitation by KCl. Prima facie, it appears that similar interactions are involved in both cases. DeGrado et al. (1981) found $\Delta G = 4$ kcal/mol for the tetramerization of melittin from the concentration dependence of θ. This is very close to our estimate for the aggregation process induced by 1 M KCl. The formation of melittin tetramer, i.e., of a species of definite composition, implies a definite structure. Also, it is the tetramer which is the highly soluble species. It is possible that the covalent modifications prevent formation of the tetrameric structure. Since melittin has so large a proportion of hydrophobic side chains, the modified melittin may form a different, insoluble aggregate to minimize hydrophobe–water contacts.

References

Ackers, G. K. (1975) *Proteins, 3rd Ed. 1.*

Bello, J. (1977) *J. Theor. Biol. 67*, 335.

Bello, J. (1978) *Int. J. Pept. Protein Res. 12*, 38–41.

Bello, J., Iijima, H., & Kartha, G. (1979) *Int. J. Pept. Protein Res. 14*, 199–212.

Brown, L. R., Lauterwein, J., & Wüthrich, K. (1980) *Biochim. Biophys. Acta 622*, 231–244.

Dawson, C. R., Drake, A. F., Helliwill, J., & Hider, R. C. (1978) *Biochim. Biophys. Acta 510*, 75–86.

DeGrado, W. F., Kezdy, F. J., & Kaiser, E. T. (1981) *J. Am. Chem. Soc. 103*, 679–681.

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

Goren, H. J., McMillin, C. R., & Walton, A. G. (1977) *Biopolymers 16*, 1527–1540.

Greenfield, N. J., & Fasman, G. D. (1969) *Biochemistry 8*, 4108-4116.
Habermann, E. (1972) *Science (Washington, D.C.) 177*, 314-322.
Habermann, E., & Jentsch, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 37-50.
Habermann, E., & Kowallek, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 884-890.
Knöppel, E., Eisenberg, D., & Wickner, W. (1979) *Biochemistry 18*, 4177-4180.
Lauterwein, J., Brown, L. R., & Wüthrich, K. (1980) *Biochim. Biophys. Acta 622*, 219-230.
Lübke, K., Matthes, S., & Kloss, G. (1971) *Experientia 27*, 765-767.
Morrison, T. J. (1952) *J. Chem. Soc.*, 3814-3818.
Morrison, T. J., & Billett, F. (1952) *J. Chem. Soc.*, 3819-3822.
Schroeder, E., Lübke, K., Lehmann, M., & Beetz, I. (1971) *Experientia 27*, 764-765.
Talbot, J. C., Dufourcq, J., deBony, J., Faucon, J. F., & Lussan, C. (1979) *FEBS Lett. 102*, 191-193.
Tanford, C. (1980) *The Hydrophobic Effect*, 2nd ed., p 11, Wiley, New York.
Terwilliger, T. C., Weissman, L., & Eisenberg, D. (1982) *Biophys. J.* (in press).

Conformational Equilibrium of Demetalized Concanavalin A†

Rodney D. Brown, III,* Seymour H. Koenig, and C. Fred Brewer

ABSTRACT: Concanavalin A (Con A) is known to exist in two conformations [Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883-3896] that differ in their metal ion and saccharide binding properties. The conformation that binds metal ions tightly, and which is associated with saccharide binding, has been designated as "locked" and that which binds metal ions only weakly as "unlocked". In the presence of excess metal ions, such as Mn^{2+} and Ca^{2+}, essentially 100% of the protein is in the locked conformation. The scheme proposed to explain these effects [Koenig, S. H., Brewer, C. F., & Brown, R. D., III (1978) *Biochemistry 17*, 4251-4260] predicts an equilibrium between these conformations for the apoprotein. By monitoring the solvent proton relaxation dispersion as equimolar concentrations of Mn^{2+} and Ca^{2+} are titrated, at 5 °C, into an apo-Con A solution that had been equilibrated at 25 °C, we find that 12.5% of the apoprotein is in the locked conformation, corresponding to an energy separation of 1.2 kcal mol^{-1}. We also show that these conformations can be separated by column chromatography at 5 °C and that the 100% unlocked form prepared in this way returns to the expected equilibrium mixture when kept at 25 °C.

Concanavalin A (Con A),[1] a metalloprotein isolated from the jack bean (*Canavalia ensiformis*), is a dimer below pH ~6 of molecular weight 54000 (McKenzie et al., 1972; Senear & Teller, 1981). For some time it has been known that full saccharide binding and agglutination properties of Con A require the presence of divalent cations (Yariv et al., 1968; Kalb & Levitzki, 1968; Agrawal & Goldstein, 1968; Inbar & Sachs, 1969). There are two cation binding sites per monomer, a site S1 that binds a variety of divalent transition-metal ions, including Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+}, and a site S2 that binds Ca^{2+} and Cd^{2+} (Kalb & Levitzki, 1968; Shoham et al., 1973). Recent evidence indicates that Ca^{2+} can also bind at S1 (Harrington & Wilkins, 1978; Koenig et al., 1978) and Mn^{2+} at S2 (Brown et al., 1977). S2 is not formed until S1 is occupied (Shoham et al., 1979), and occupation of both sites is believed necessary for full saccharide binding (Agrawal & Goldstein, 1968) and agglutination activity (Inbar & Sachs, 1969), though the stoichiometry of activation of Con A by Mn^{2+} has been questioned by Christie et al. (1980).

It is known that there are two conformations of Con A separated by a high energy barrier, about 22 kcal mol^{-1}, with their ground-state energies differing by only a few kilocalories per mole, the sign of this difference depending on the occupancy of S1 and S2 by metal ions (Brown et al., 1977). A major distinction between the two conformations is that the one associated with fully metalized protein (the "native" protein) has a far greater affinity for metals than the other. It was named, accordingly, the "locked" conformation, and the other, "unlocked".

It was conjectured by Brown et al. (1977) that the saccharide binding and agglutination activity of the fully metalized protein were properties associated with the locked conformation as well as with the metal ion content. The correlation of these activities with the presence of metal ions was considered to derive from the fact that metal ions lower the energy of the locked relative to the unlocked conformation, making it the predominant metalized form at equilibrium. This conjecture was subsequently verified by Harrington & Wilkins (1978) and Koenig et al. (1978). Using different methods, they showed that demetalized Con A in the higher energy, metastable, locked conformation binds saccharide quite

† From the IBM Thomas J. Watson Research Center, Yorktown Heights, New York 10598 (R.D.B. and S.H.K.), and the Department of Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461 (C.F.B.). *Received August 7, 1981.* This work was supported in part by Grant CA-16054, awarded by the National Cancer Institute, Department of Health, Education, and Welfare.

[1] Abbreviations: Con A, concanavalin A with unspecified metal content; P, AP, and BAP, apo-Con A, the binary complex of Con A with metal ion A at binding site S1, and the ternary complex of Con A with metal ion B at binding site S2 and A at S1, respectively, all with the protein in the "unlocked" conformation; the suffix L (e.g., BAPL) indicates the analogous molecule in the "locked" conformation; the prefix S (e.g., SBAPL) indicates bound saccharide; M and C, Mn^{2+} and Ca^{2+} ions, respectively; EDTA, ethylenediaminetetraacetic acid; α-MDM, methyl α-D-mannopyranoside; NMRD, nuclear magnetic relaxation dispersion.

0006-2960/82/0421-0465$01.25/0