THE STRUCTURE OF CRYSSTALLINE AND MEMBRANE-BOUND GRAMICIDIN A
BY VIBRATIONAL ANALYSIS
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The results of normal mode calculations on the $\beta^{4.4}$, $\beta^{6.3}$, $\alpha\beta^{5.6}$, and
$\alpha\beta^{7.2}$ structures of gramicidin A are compared with infrared and Raman
spectra of crystalline native, crystalline Cs$^+$-bound, and vesicle-bound
gramicidin A. The observed frequencies and frequency splittings are in good
agreement with an assignment of $\alpha\beta^{5.6}$, $\alpha\beta^{7.2}$, and $\beta^{6.3}$ structures,
respectively, to the gramicidin A molecules in the above three systems.

Gramicidin A (GA), an antibiotic polypeptide isolated from Bacillus
brevis, is a linear alternating L,D pentadecapeptide which facilitates the
passive transmembrane transport of ions in biological membranes and synthetic
lipid bilayers (1,2). This transmembrane channel has been shown to consist of
two molecules of GA (3-8). The detailed structure of the dimer has been under
investigation for a number of years, with two main models having been pro-
posed: a formyl end-to-formyl end hydrogen-bonded association of single $\beta^{6.3}$
helices (9-11), and a double-stranded $\beta$-helix structure with a most probable
antiparallel alignment of hydrogen-bonded GA molecules (12-14). Conforma-
tional energy calculations on single- and double-stranded $\beta$-helical poly(L,D-
alanine) (15-17) have yielded almost identical conformational energies for
these two kinds of helices, thus providing no basis for the preference of one
model over the other. In a recent calculation on the head-to-head dimer with
side chains explicitly included, it was shown that left-handed $\beta^{6.3}$ helices
are of lower energy than right-handed (18).

Different experimental studies have indicated that GA can adopt single-
stranded or double-stranded structures depending on the environment. Diffra-
cion studies on GA crystallized in its native and various cation-bound forms
and low-resolution Fourier maps (21), are consistent with a helical molecule, but no detailed crystal structure has yet been published. However, the x-ray studies do show that the length of the dimer decreases from 32 to 26 Å, and the diameter of the cylinder formed by the peptide main chain atoms increases from 5 to 6.8 Å, on binding Cs⁺ ions (20). From ¹³C and ²³Na NMR studies and CD studies on GA and synthetic selectively ¹³C-enriched GA (22–25), a head-to-head dimer structure has been inferred for the active transmembrane channel. A recent CD study (26) has emphasized the differences in structure between GA in membranes (a head-to-head dimer) and in organic solvents (a double-stranded helix), and a two-dimensional NMR study (27) has shown that a double-stranded +β⁵.⁶ helix exists in dioxane.

There have been a number of infrared (IR) (12,28–35) and Raman (26,28,35,36) studies on GA and its analogs. In some of the IR work (31,33,34), D₂O was used in order to avoid the water absorption in the amide I region (1600–1700 cm⁻¹), and neglect of the effect of deuteration of the peptide group on this frequency (see below) leaves these interpretations questionable. In one of the IR studies (34) the measurements were made on dried membrane preparations, leaving open the question of whether the structure in a dried specimen is the same as in an aqueous membrane environment, even assuming that the GA is still within the membrane. In all cases, except for our initial report (35), interpretations were based on qualitative correlations or approximate coupling calculations (30).

**METHODS**

In order to provide a reliable interpretation of the vibrational spectra of GA in terms of structure, we have used the powerful approach of normal coordinate analysis (37). We have done normal mode calculations (38) on all structures that are consistent with the observed dimer length and channel radii, viz., single-helical +β⁴.⁴ and +β⁵.³ and double-helical +β⁵.⁶ and +β⁶.⁴. The normal modes were computed with the side chain approximated by a point mass equal to CH₃, using a force field developed for β-poly(L-alanine) in this approximation (39). Transition dipole coupling (40,41) was included for amide I and amide II modes, using calculated, not assumed (30), eigenvectors. The calculated frequencies for amide I modes, expected to be accurate to about ± 5 cm⁻¹ (42), are given in Table 1, and are compared to IR and Raman data in the crystalline state (35) and in vesicles.
Table 1. Observed and Calculated Amide I Frequencies (in cm⁻¹) for Single- and Double-StrandedGramicidin A Structures

<table>
<thead>
<tr>
<th>Structure</th>
<th>Calculateda</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v(IR)b</td>
<td>Δυc</td>
</tr>
<tr>
<td>β4.4</td>
<td>1631</td>
<td>13</td>
</tr>
<tr>
<td>β6.3</td>
<td>1643</td>
<td>11</td>
</tr>
<tr>
<td>++β5.6</td>
<td>1636</td>
<td>39</td>
</tr>
<tr>
<td>++β7.2</td>
<td>1632</td>
<td>45</td>
</tr>
</tbody>
</table>

aRef. 38. bStrong parallel-polarized IR mode (38). cDifference between strong parallel and strongest (though weaker) perpendicular IR modes (38). dRange of IR-weak frequencies; Raman mode in crystalline forms observed at mid-point of range. eEstimated from observations on vesicles (31,34) (see text). fThis work. gRef. 35.

RESULTS AND DISCUSSION

In the IR spectrum of native GA crystals there is a strong amide I band at 1638 cm⁻¹ with a shoulder at -1680 cm⁻¹, corresponding to a splitting, Δυ, of -42 cm⁻¹ (35). On binding Cs⁺ or K⁺ the strong band shifts down to 1632 cm⁻¹ and the higher frequency band becomes a distinct peak at 1685 cm⁻¹, giving Δυ = 55 cm⁻¹ (35). The calculated frequencies of the strong (parallel polarized) bands, their shifts on ion binding, and the magnitudes and changes in Δυ (the difference between the weak high frequency perpendicular mode and the strong low frequency parallel mode) are in excellent agreement with an assignment of the ++β5.6 structure to crystalline native GA and the ++β7.2 structure to crystalline ion-bound GA. An assignment to single-stranded helices is precluded primarily by the much smaller Δυ calculated for these structures, but also by the poorer agreement with the observed strong IR and Raman modes (note that the latter are observed at about the mid-point of the range of calculated IR-weak frequencies). It should be mentioned that the ++β5.6 structure, with a channel radius for unhindered ion passage of 0.9 Å, would be unlikely to hold a Cs⁺ ion, whose ionic radius is 1.48 Å, whereas the ++β7.2 structure, with its comparable channel radius of 2.0 Å, could easily do so. We also wish to note that a previous calculation of Δυ (30) gave values (64 cm⁻¹) for the double-stranded helices much higher than ours, a result of
assuming that the eigenvectors for the amide I modes correspond to those of an
infinite β-sheet structure rather than to those of the actual helical struc-
ture, which are obviously different and which we obtain from our normal mode
calculations.

The situation for GA in lipid vesicles is more complicated because of the
assignment and interpretation of the IR amide I mode. The measurements on a
liposome suspension (31) and those on a film dried from vesicles (34), which
gave amide I bands at 1634 and 1633 ± 1 cm⁻¹, respectively, were both done
after D₂O treatment. It is well known that deuteration of the peptide group
causes the amide I frequency of β structures to decrease (43,44), and these
observations indicate a likely decrease of about 6 cm⁻¹ in the present case.
Thus, the unshifted amide I mode for GA in vesicles should be at -1640 cm⁻¹.

As is indicated by the calculations, the distinction between β₆.₃ and ++β₅.₆
is therefore difficult to make on the basis of the strong IR mode alone,
although the absence of a higher frequency component in the liposome system
(31), which CD spectra (31) show to be in the conducting state (34), favors
the β₆.₃ structure.

Since the calculations indicate that the Raman mode should be at a sig-
ificantly lower frequency in single-stranded than in double-stranded struc-
tures, we have examined the Raman spectra of GA in vesicles. We have prepared
such vesicles in H₂O by the method of Urry et al. (34), and the Raman spectra
are shown in Fig. 1. The spectrum of vesicles alone is shown in Fig. 1a, the
1640 cm⁻¹ band being due to H₂O. In the early stages of incorporation (Fig.
1b), a band is seen at 1665 cm⁻¹, accompanied by a weak band at 1654 cm⁻¹. In
the conducting state (Fig. 1c), the former band is absent and only the 1654
cm⁻¹ band is present. When the vesicles are dried (Fig. 1d), a strong band
returns at 1671 cm⁻¹, with a weak band remaining at -1650 cm⁻¹.

These results can be interpreted as follows. The initial structure of GA
is ++β₅.₆ (possibly with some ++β₇.₂ if complexing with Na⁺ occurs), having a
characteristic Raman band near 1667 cm⁻¹. As incorporation proceeds, the
structure converts to β₆.₃ with a characteristic Raman band near 1654 cm⁻¹.
Figure 1. Raman spectra (Spex 1403) of (a) vesicles in H$_2$O, (b) gramicidin A in vesicles after 10 min. of incubation at 45°C, (c) gramicidin A in vesicles after 8-15 hr. of incubation at 60°C, (d) dried gramicidin A - vesicles. ($\lambda_{exc} = 5145$ Å, laser power -100 mW, resolution -2 cm$^{-1}$, co-addition of at least 20 scans per spectrum.)

(this is preferable to an assignment to $\beta^{4,4}$, not only because of the somewhat better agreement with the calculated Raman mode but also because of the much better agreement with the strong IR mode). This structure has also been proposed from NMR studies (22,25). On drying, the GA converts back substantially to the $\#\beta^{5,6}$ (or possibly $\#\beta^{7,2}$) structure, although it is not known whether or not the GA is still incorporated in the membrane. This accounts for the complex band structure in the dried film (34), whereas a simple IR band is seen for the liposome system (31).
Normal mode calculations thus provide a powerful base for analyzing the structure of gramicidin A from infrared and Raman spectra.

REFERENCES AND NOTES

45. This is paper 28 in a series on "Vibrational Analysis of Peptides, Polypeptides, and Proteins," of which (37) is paper 27. This research was supported by a fellowship from the Macromolecular Research Center at The University of Michigan (to V.M.N.) and by NSF grants PCM-8214064 and DMR-8303610.