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Structure of an Eu^{3+}–gramicidin complex in methanol–water solutions studied by laser excitation spectroscopy

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Abstract

Two complexes of europium ion and gramicidin A were stabilized with increasing water mole fraction in a water–methanol mixture. These complexes were detected using environment-sensitive laser excitation spectroscopy. For 0–0 transitions in the 579 nm region, the single absorption line for the aqueous ion at 578.9 nm was gradually replaced by two narrow lines at 579.1 and 579.4 nm, respectively. The amplitude and bandwidths of these lines were consistent with intimate, discrete site binding while the lifetimes observed following selective excitation to each of these new lines were a signal of the loss of two and three water molecules, respectively, from the inner hydration sphere on formation of the ion–gramicidin complex. The local ligand field symmetry was deduced from level splitting for the $^7F_0 \rightarrow {}^5D_2$ transitions in the 464 nm region. The eight absorption lines remaining after elimination of known aqueous ion lines were resolved into a triplet and a pentet for the respective gramicidin binding sites. The triplet was generated by ions occupying a binding site having C_{3v} ligand field symmetry. These data, in concert with circular dichroism and other fluorescence data, restrict the number of possible ion–gramicidin conformers in methanol–water solutions.

Keywords: Europium; Gramicidin; Laser excitation spectroscopy; Helix

1. Introduction

The gramicidin A molecule with its alternating D and L amino acid residues can assume a variety of ordered conformations. In lipid bilayer membranes, the molecule is a right-handed single helix that dimerizes via hydrogen bonding of the two formyl termini to produce an ion-conducting channel [1–3]. The channel lumen has a diameter of 0.4 nm and conducts most univalent metal cations. The kinetics of association and dissociation of single dimeric channels can be monitored electrochemically [4].

Polyvalent ions in the bathing solutions can reduce the univalent cation flow through gramicidin channels. This block is partial and potential dependent and has been attributed to rapid association and dissociation kinetics of the polyvalent ion [5]. The ethanolamine terminus of the operating gramicidin channel is the only region of the molecule accessible to ions from the bathing solution. Experiments with Eu^{3+} and a tunable narrow-band laser, however, show no observable binding to gramicidin molecules in vesicle membranes [6]. The hydrophobic character of the membrane surrounding the dimeric gramicidin

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channel may act to limit binding to this conformation [7].

Both the conformation and the ion-binding properties of the molecule in solution can differ from those of the molecule in the membrane. Gramicidin molecules in homogeneous, ion-free solutions have a variety of monomeric and dimeric conformations [8,9] with relative populations that are a sensitive function of solvent composition and temperature. The dimeric conformers are parallel and antiparallel double helices [10,11] that may revert to single helix with increasing solvent polarity [12].

Ion-induced conformational changes in gramicidin are observed with circular dichroism (CD) [13], infrared [10], and nuclear magnetic resonance (NMR) spectroscopy [14]. In hydrophobic solvents, the univalent ions Cs^+ and Li^+ bind to gramicidin to produce changes in both the handedness and pitch of the molecule. X-ray studies of a Cs^+–gramicidin complex reveal a left-handed, antiparallel double helix [15,16] that binds the Cs^+ ions.

All helical structures of gramicidin are sparingly soluble in polar solvents like water because the gramicidin amino acid side chains are non-polar. However, theoretical calculations indicate that a head-to-head dimer of single helices might be surrounded by an average of about 15 water molecules suggesting that this conformer might be stable in water [17].

Specific binding environments for the lanthanide ions, Eu^{3+} and Tb^{3+}, can be characterized using environment-sensitive laser ion excitation spectroscopy [18,19]. The absorption bandwidths for the 0–0 $^7F_0 \rightarrow {}^5D_0$ transitions for aquated Eu^{3+} ion can be as narrow as 8 cm^{-1}. Both the absorption maximum and the bandwidth change when the local environment of the ion changes. Narrow-band laser excitation spectroscopy for the 0–0 transition in the 579 nm region produces resolvable spectral lines for each distinct ion binding environment.

Since ions in a specific environment can be selectively excited with a narrow-band laser, the properties of the ion in that environment can be studied by observing environment-sensitive changes in the emission properties of the ion. In aqueous solutions, the Eu^{3+} ions are surrounded on average by 8.3 water molecules [20]. Some water molecules can be displaced from the ionic hydration shell to permit ligand binding. The lifetime of an ion in a specific environment provides a measure of the residual waters on the ion in that environment [18], making it possible to estimate the number of water molecules lost when the aquated ion binds to a ligand. Additional information is generated by laser excitation studies of the $^7F_0 \rightarrow {}^5D_2$ transition in the 464 nm region. The splitting of this five-fold degenerate level provides a measure of the local ligand field symmetry at each specific ion binding site [21].

In this paper, we demonstrate the application of environment-sensitive Eu^{3+} excitation spectroscopy to characterize the binding of Eu^{3+} ions to gramicidin in water–methanol solutions. New absorption lines signal new ion binding environments. The lifetime of the resulting $^5D_0 \rightarrow {}^7F_2$ emission is used to predict the changes in the Eu^{3+} hydration shell structure upon binding. The analysis of the splitting pattern of the five-fold degenerate $^7F_0 \rightarrow {}^5D_2$ Eu^{3+} absorption line describes the binding site local field symmetry. Additional information associated with the structure of possible gramicidin conformers is obtained from circular dichroism spectroscopy and conventional broad-band Eu^{3+} excitation spectra. The data characterize the Eu^{3+}–gramicidin conformer in polar media with highly charged cations.

2. Materials and methods

2.1. Samples

Gramicidin D (Sigma) was used without further purification as a 1 mM solution in methanol. A standard 1 mg/ml (6.6 mM) solution of $Eu(NO_3)_3$ (Aldrich) was the Eu^{3+} source in all experiments. The samples were prepared by mixing appropriate aliquots of these solutions with methanol and/or water to make four solutions with different water mole fractions (0.47, 0.52, 0.57 and 0.63). The concentrations of Eu^{3+} and gramicidin were constant for all samples with concentrations of 1.39 and 0.37 mM (3.7:1 molar ratio), respectively.

2.2. Instrumentation

The spectrometer source is a Molectron D laser system with a tunable dye laser pumped by a pulsed

nitrogen laser. Coumarin 480 in ethanol and rhodamine B borate in trifluoromethanol were selected as laser dyes for maximum beam intensity in the 464–468 and 578–580 nm spectral regions, respectively. The laser beam passes twice through a 1-cm quartz cuvette while fluorescence at 614 nm is collected from two opposing cuvette faces using a mirror and lens arrangement. The emission is selected by a Bausch & Lomb monochromator (1.8 nm/mm; 1.3 nm bandwidth) and a Corning cut-off filter (CS2-62). The photons are detected with an Amperex XP1002 red-sensitive photomultiplier tube. A Pacific Photometrics photon counting module and a Scientific Solutions interface transmit photon counts to an IBM AT computer. The interface also provides clock pulses to a stepper motor to change laser wavelengths in increments of 0.02 nm/step. The deconvolution of overlapping spectral bands was performed using Sigmaplot scientific software based on a standard Marquardt–Levenberg algorithm with preset initial parameters.

Eu^{3+} emission lifetimes were measured with a Northern Scientific multichannel scaler with a channel dwell time of 8.3 μs. Lifetimes are observed to within ± 2 μs.

Broadband Eu^{3+} excitation spectra were recorded with an SLM 8000 fluorometer tuned for emission at 614 nm. Circular dichroism spectra were recorded on a Jasco spectrometer with a Sproul Scientific SS 15-2 CD modification and a xenon lamp as an excitation source. The baseline was recorded for every solvent used and then subtracted from the actual data to correct for any residual elipticity.

3. Results

Laser excitation spectra for Eu^{3+} and gramicidin in water–methanol mixtures with water mole fractions are shown in Fig. 1. The methanol solution with a trace of water has a narrow absorption band peaking at 578.8 nm with a bandwidth of 10 cm^{-1}. As the water mole fraction increases, two new narrow (2 cm^{-1}) bands are observed at 579.1 nm and 579.4 nm in concert with a decrease in the band amplitude of the aquated ions at 578.8 nm. The new bandwidths are narrower than those observed for the Eu^{3+}–satellite tobacco necrosis virus (STNV) complex by a factor of three [21]. The ratio of the peak intensities of the two new bands is constant at all mole fractions. The three bands in Fig. 2 can be

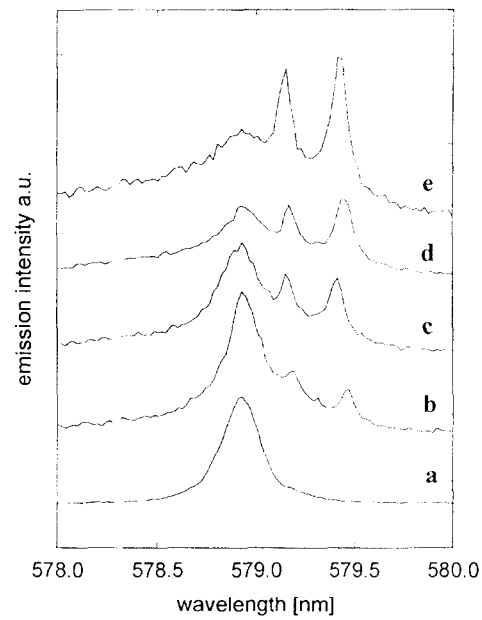

Fig. 1. $^7F_0 \rightarrow {}^5D_0$ Eu^{3+} excitation spectra with gramicidin in water–methanol solutions with different water mole fractions: (a) methanol, (b) 0.47, (c) 0.52, (d) 0.57, (e) 0.63.

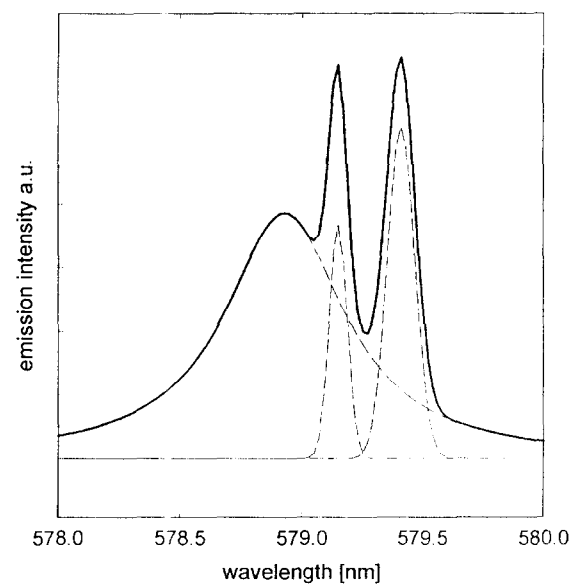

Fig. 2. Curve fitting separation of the three $^7F_0 \rightarrow {}^5D_0$ absorption bands for Eu^{3+} complexed with gramicidin in water–methanol mixture with $X_w = 0.63$.

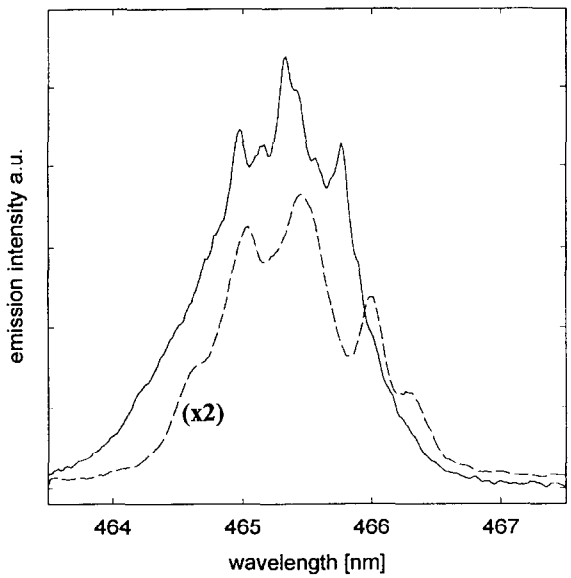

Fig. 3. $^7F_0 \rightarrow {}^5D_2$ Eu^{3+} excitation spectra of a 1.39 mM solution of $Eu(NO_3)_3$ in water (dashed line) and Eu^{3+} complexed with gramicidin in water–methanol mixture with $X_w = 0.63$ (solid line).

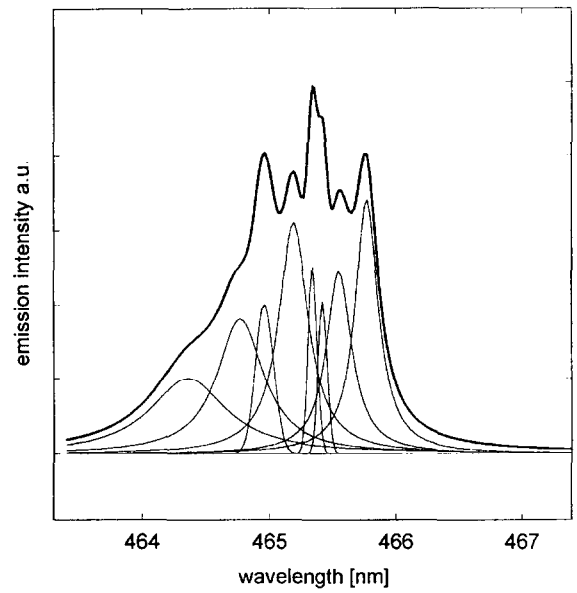

Fig. 4. Curve fitting separation of the eight $^7F_0 \rightarrow {}^5D_2$ absorption bands for Eu^{3+} complexed with gramicidin in water–methanol mixture with $X_w = 0.63$ (detailed description in text).

resolved into two Gaussian (579.1 and 579.4 nm) and a single Lorentzian peak (578.8 nm) using the deconvolution algorithm.

The 614 nm emission decay for the methanol solution is a single exponential with a 104 μs lifetime. For $X_w = 0.63$, the lifetime following excitation of the aqueous ion is 104 μs. Eu^{3+} ions at the distinct binding environments characterized by excitations at 579.1 and 579.4 nm give lifetimes of 160 and 185 μs, respectively. The fully hydrated Eu^{3+} ion contains on the average 8.3 water molecules in its inner hydration shell [20]. The lifetime correlation table of Horrocks and Sudnick [18] predicts the loss of two and three water molecules, respectively, from the hydration sphere of the Eu^{3+} ion for these two distinct binding environments.

In the 466 nm region ($^7F_0 \rightarrow {}^5D_2$), the spectrum of the solvated ion (five peaks) is scaled and subtracted from the total spectrum (Fig. 3) to give the multiline spectra for the two anticipated binding environments predicted from analysis in the 579 nm region. The scaling coefficient is equal to the ratio of the absorption intensity of the aqueous peak at 579 nm (Fig. 1e) to the intensity observed for the solution of Eu^{3+} and gramicidin in pure methanol (Fig. 1a). The intensities of the residual, narrowed spectral bands are increased relative to the solvated ions by a factor of three. The deconvolution produces eight

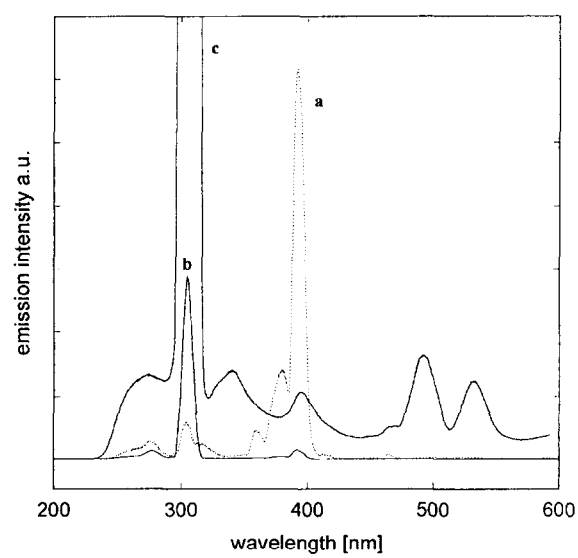

Fig. 5. Broad-band excitation spectra of Eu^{3+}: (a) 1.39 mM solution of $Eu(NO_3)_3$ in water, (b) Eu^{3+} without gramicidin in water–methanol mixture with $X_w = 0.63$, (c) Eu^{3+} complexed with gramicidin in a water–methanol mixture with $X_w = 0.63$ (low-intensity structure).

separate absorption lines that resolve into a triplet with Gaussian peaks at 465.0, 465.3 and 465.4 nm and a pentet with Lorentzian peaks at 464.4, 464.8, 465.2, 465.6 and 465.8 nm. The two different band-shapes simplify attribution of the peaks. Since the two binding absorption peaks for Eu^{3+} with gramicidin are Gaussian in the 579 nm region, the 464 nm region peaks for the bound ions might also be expected to be Gaussian. However, the fitting procedure using the three Gaussian and five Lorentzian peaks (Fig. 4) gives the lowest unnormalized squared sum of deviations (125.7) relative to 435.5 for eight Gaussian peaks with higher sums for other combinations. The three-fold splitting is attributed to an ion binding site consistent with C_{3v} symmetry while the five-fold splitting suggests a site with less symmetry.

Near-ultraviolet excitation spectra for aqueous Eu^{3+}, the methanol Eu^{3+}–gramicidin and methanol–water Eu^{3+}–gramicidin system ($X_w = 0.63$), taken on an SLM spectrofluorometer, are shown in Fig. 5. The emission was monitored for the Eu^{3+} $^5D_0 \rightarrow {}^7F_2$ radiative transition at 614 nm. In the presence of gramicidin, the increasing mole fraction causes a 100 × enhancement of the band intensity at 304 nm and a decrease in emission intensity for the solvated ion at 394 nm.

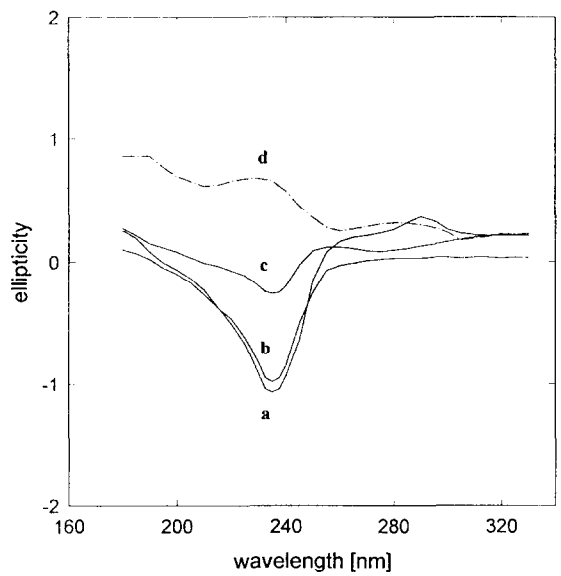

Fig. 6. Circular dichroism spectra (uncalibrated) of (a) gramicidin in methanol, (b) gramicidin with Eu^{3+} in methanol, (c) gramicidin in a water–methanol mixture with $X_w = 0.63$ without Eu^{3+}, (d) Eu^{3+} complexed with gramicidin in a water–methanol mixture with $X_w = 0.63$.

CD spectra for the gramicidin in methanol in the range 190–300 nm (Fig. 6) show a strong negative CD peak at 240 nm. The peak intensity does not change upon addition of Eu^{3+}. However, addition of water to the Eu^{3+}-free solution changes the CD spectrum.

4. Discussion

Environment-sensitive Eu^{3+} excitation spectroscopy gives new data to characterize an ion binding site in a simple protein system. It establishes the number of distinct ion binding sites on the protein. For gramicidin in solution with a large water mole fraction, we observe two new bands at 579.1 and 579.4 nm that are not observed in methanol solution. With a tunable laser, ions in either of these binding environments can be selectively excited to probe the nature of that specific ion binding site. The lifetimes of these selectively excited ions show that the ions bind to two different protein sites with the loss of two and three water molecules, respectively. This minimal ligation is most consistent with binding either to a terminus of an extended conformer or in the interior of a water-filled conformer.

The single helical conformer of gramicidin has been observed in highly polar solvents such as DMSO [12]. The single helix excludes polyvalent ions when it forms channels in membranes. If this conformer exists in water–methanol solutions, binding is expected to occur to the oxygen atoms located outside the lumen or pore of the helix. The termini of the single helical conformer have oxygens that can displace two and three water molecules, respectively. A double helix conformer, on the other hand, could bind the ion at either an exterior terminus (or termini) or in the interior region of the double helix so that only two or three water molecules are displaced from the inner hydration sphere of the ion.

The spectral lines associated with binding to gramicidin are narrowed relative to the aqueous ion lines. These data suggest intimate binding to the two or three exposed sites.

The splitting of the five-fold degeneracy of the excited $^5D_2$ state gives the local symmetries of the

ions at their protein binding sites and provides constraints on possible ion–gramicidin conformations. The eight absorption lines remaining after subtraction of aqueous ion absorption lines resolve into a triplet and a pentet. The ions generating the pentet are in an environment having little or no symmetry. The ions generating the triplet, by contrast, require a local C_{3v} symmetry that could be consistent with the ion bound at a terminus of the single helix or either the interior or a terminus of a double helix conformation. There is evidence, for example, that Cs^+ binds within a double helix [13]. Either type of binding is consistent with the predicted loss of only two or three water molecules from the ion's inner hydration sphere.

The conventional Eu^{3+} excitation spectroscopy shows the appearance of an increased amount of ion binding as the solution polarity increases. It corroborates the observation that the concentration of ion binding conformer is minimal in pure methanol.

The CD spectrum of gramicidin in methanol has a large negative band near 238 nm, which decreases in intensity with increasing water mole fraction and becomes positive after Eu^{3+} ions are introduced. These changes may be due to either an increase in the concentration of the right-handed parallel double helix with net positive dichroism in this region [10] or the appearance of a new conformer stabilized by Eu^{3+}. The most viable alternative is a single helix since this conformer in vesicle membranes gives a positive CD absorption at this wavelength as well. The appearance of only two new Eu^{3+} absorption lines in the 579 region and the constant ratio of their intensities in solutions with different water mole fractions are consistent with one dominant Eu^{3+}–gramicidin conformer rather than an equilibrium mixture of conformers.

With this new data, it is impossible to distinguish the right-handed parallel double helix from the single helix. There is strong evidence for the double helix [10] and its ion complexes [13] in these polar solvents. However, the ends of the single helix have three and two oxygens, respectively, that could bind Eu^{3+} with the loss of three and two waters. The three oxygens at the formyl terminus have C_{3v} symmetry. Computer analysis of the parallel double helix is necessary to determine whether the double helix has an ion binding site of the proper local symmetry.

Laser excitation spectroscopy points to Eu^{3+} ion binding to two distinct sites on a molecular conformation that is stabilized with increasing water mole fraction and, perhaps, by the ion itself. It establishes the local symmetry of the binding site and the net loss of inner sphere water molecules during binding. This information narrows the possible gramicidin conformations in these water–methanol solutions.

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