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Conformation of Gramicidin A in Water: Inference from Analysis of Hydrogen/Deuterium Exchange Behavior by Matrix Assisted Laser Desorption Ionization Mass Spectrometry

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Abstract: *Gramicidin A (the major component of gramicidin D) is a highly hydrophobic peptide with very little solubility in water. Hence, the conformation of this peptide has been extensively investigated in organic solvents and model membranes, but not in water. The peptide adopts a $\beta^{6.3}$-helical conformation in the monomeric and dimeric forms. We have investigated the conformation of gramicidin A in water by monitoring hydrogen–deuterium exchange by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Our results indicate that gramicidin A is monomeric and exists in a highly folded conformation. The metal ion bound forms are clearly discernible in the monomers. The presence of the dimeric form is not observed. It is unlikely this is due to the operating conditions or the method used, as both hetero- and homodimers in gramicidin D are detected when methanol is used as a solvent. The present study also establishes that the linear gramicidins retain a history of solvent environment when ions are generated by matrix-assisted laser desorption ionization and analyzed by time-of-flight.* © 2005 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 80: 708–713, 2005

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Keywords: *peptide conformation; matrix assisted laser desorption ionization mass spectrometry; hydrogen bonds; peptide aggregation; hydrophobic peptides*

INTRODUCTION

The channel-forming peptide gramicidin A (the major component of gramicidin D) has been the subject of extensive structural investigations.[1–3] The peptide is composed of only hydrophobic amino acids. Both the N- and C-termini are blocked. Hence, the peptide has very low solubility in water, and solution conformational studies have been confined to organic solvents[4–8] and membrane environments.[9–12] In pure alcohols and 1,4-dioxane, the peptide adopts parallel, antiparallel, left-handed, or right-handed double-stranded helical structures that intertwine with each other. In polar solvents such as dimethylsulfoxide and 2,2,2-trifluoroethanol, the peptide is presumed to adopt a $\beta^{6.3}$-helical conformation in the monomeric form.[4,5] It has also been

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proposed that there is an equilibrium between a disordered structure and a monomeric $\beta^{6.3}$-helical conformation in solution, in polar solvents.[13] The structures of gramicidin A in the uncomplexed form and complexed with metal ions, crystallized from organic solvents, have been reported.[14–20] All the structures are intertwined double-stranded double helix dimers. Duax and coworkers have proposed that the conducting form of gramicidin A is a right-handed double-stranded double helix.[18–21] Andersen et al.,[22] Cross et al.,[23] and others[9–12] have argued that the predominant channel form is a head-to-head dimer of two single-stranded β-helices. Compression isotherms of gramicidin A in monolayers have been attributed to helical conformation at the air–water interface and the presence of aggregates.[24–26] The conformation of gramicidin has not been investigated in water, presumably due to low solubility, rendering application of nuclear magnetic resonance (NMR) or circular dichroism (CD) methods impossible. Electrospray ionization (ESI) mass spectrometry has been used to examine the conformation of gramicidin A in different organic solvents and aqueous mixtures of organic solvents.[27,28] Analysis in pure aqueous medium is not easy by ESI mass spectrometry and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry[29,30] would be more suitable for analysis from pure aqueous medium. We describe in this article hydrogen–deuterium exchange behavior of protons in gramicidin D when dissolved in water, by MALDI-TOF mass spectrometry.

MATERIALS AND METHODS

Gramicidin D was purchased from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification.

Natural gramicidin is a mixture of six variants.[31] The amino acid sequence of gramicidin A (Val–GA) is HCO–Val[1]–Gly–Ala–*Leu*–Ala[5]–*Val*–Val–*Val*–Trp–*Leu*[10]–Trp–*Leu*–Trp–*Leu*–Trp–$NHCH_2CH_2OH$. D-amino acids are in italics. In gramicidin B (GB) and gramicidin C (GC), the tryptophan at position 11 is replaced by phenylalanine and tyrosine, respectively. In 5–20% of the molecules, valine at position 1 is replaced by isoleucine. Ile–GA denotes gramicidin A in which Val[1] is replaced by Ile.

The matrix used was α-cyano-4-OH cinnamic acid (CHCA). The crude CHCA was dissolved in deuterated methanol and precipitated with D_2O. The precipitated CHCA was dissolved in CH_3CN/D_2O containing 0.1% deuterated trifluoroacetic acid. Guanidine deuterium chloride was prepared by dissolving guanidine hydrochloride in D_2O and carrying out repeated lyophilization. A stock solution of gramicidin D (1 m*M*) was prepared in CH_3OH. An aliquot of the peptide was dissolved in D_2O or CH_3OD (50-fold dilution, concentration of gramicidin = 20 pmoles/μL) to initiate hydrogen–deuterium exchange. At fixed time points, 1 μL of the solution was spotted onto the MALDI plate for analysis. Aliquot of gramicidin was added to D_2O containing guanidine deuterium chloride (4*M*) in order to initiate exchange in the presence of denaturant. Ten microliters of this solution was diluted to 20 μL and 1 μL of this diluted sample was spotted onto the MALDI plate for analysis. MALDI spectra in the reflectron mode were recorded in a Voyager DE STR mass spectrometer from Perceptive Biosystems at the Proteomics Facility in the Centre for Cellular and Molecular Biology. Average masses were calculated using the following equation: Σ (mass $\times$ intensity of peak)/Σ (intensity of total isotopic peaks).

RESULTS AND DISCUSSION

The MALDI spectrum of gramicidin D in water is shown in Figure 1. Natural gramicidin D occurs as a mixture of variants. The peaks arising from the various species are indicated in the figure. Both the metal ion complexed and the uncomplexed forms are clearly discernible. The mass separation between the isotopic peaks is 1 *m*/*z* unit, indicating that all the species are present as monomers. Hydrogen–deuterium exchange rates are sensitive indicators of protein and peptide structure. Amide protons involved in hydrogen bonding have decreased solvent accessibility and therefore exhibit slow exchange with deuterium. Hence, *m*/*z* values and isotopic distribution at varying time intervals after exchange is initiated with deuterium can give information about peptide structure and dynamics. In order to examine whether gramicidin adopts a folded structure in water, the accessibility of the exchangeable protons to deuterium was monitored. The spectra immediately after exchange was initiated and after longer time intervals are shown in Figure 2. The complex isotopic pattern as compared to Figure 1 indicates that deuterium exchange does take place but is minimal in the free as well as the metal ion bound forms. There appears to be no equilibrium between disordered and ordered conformations as observed in polar solvents.[13] There are 21 potentially labile protons in gramicidin A. Out of these, 15 are backbone amide protons, 4 corresponding to indole protons and 2 from ethanolamine. The average mass calculated as described in Materials and Methods for $[Val–GA + Na]^+$ are 1909.82 (15 min), 1910.84 (20 h), and 1909.52 (70° for 30 min). On comparison with the calculated *m*/*z* of 1905.06 for $[Val–GA + Na]^+$, the data indicate that only $\sim$5 protons exchange with deuterium. It is likely that these correspond to the proton in ethanolamine OH and four indole protons. The backbone amides are clearly involved in strong intramolecular hydrogen bonds as no exchange is observed even after 20 h. Even heating to 70°C did not result in exchange.

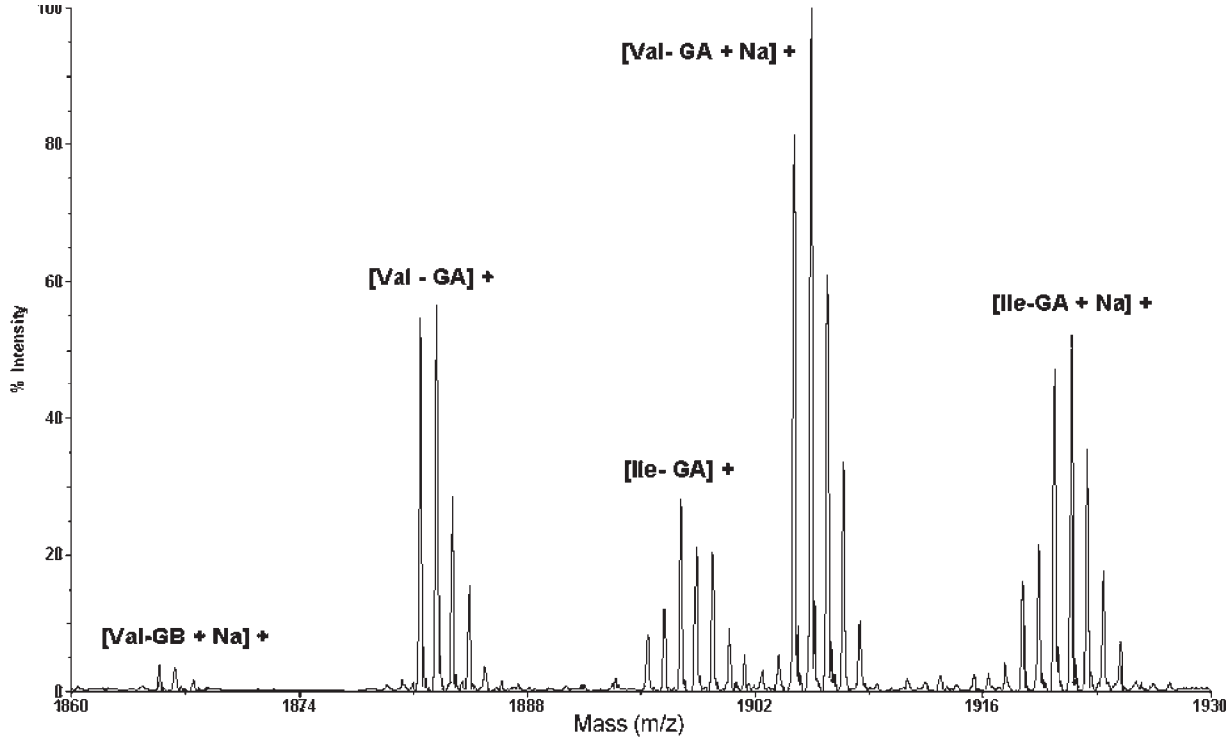

FIGURE 1 MALDI-TOF mass spectrum of gramicidin D dissolved in water using CHCA as matrix. The charge state separation shows that the species are monomeric. The abbreviations used for the peaks are Val–GB for Val–gramicidin B, Val–GA for Val–gramicidin A, and Ile–GA for Ile–gramicidin A. The abbreviations with Na^+ denote metal ion bound forms.

The absence of hydrogen–deuterium exchange was not due to the method employed for initiation of exchange and detection as almost complete hydrogen –deuterium exchange was observed with the peptides used for calibration, i.e., PPGFSPFR, DRYYIHPFHL, and EGVNDNEEGFFSAR. Since only minimal exchange was observed even on heating, hydrogen–deuterium exchange was attempted in the presence of the denaturant guanidine hydrochloride where hydrogens were replaced with deuterium. The concentration of denaturant needed to unfold the peptide was first ascertained by fluorescence spectroscopy (Figure 3). The emission spectra, as a function of the denaturant concentration, indicate a red shift and a decrease in fluorescence intensity that stabilizes at a 4*M* concentration of the denaturant. Hence, deuterium exchange was examined at this concentration. The isotopic distribution observed in Figure 4 as compared to the pattern shown in Figure 2 indicates that there is partial unfolding, resulting in exchange of some more amide protons in the presence of the denaturant. However, there is still a population of peptides in which the exchange is minimal. Even in the presence of the denaturant, both the free and metal ion bound forms are discernible. Although gramicidin A has very low solubility in water, the detection of signals clearly indicates that aggregates resistant to ionization by MALDI are not formed on drying. Was this the case, no signals would have been detected. It is thus evident that linear gramicidins do adopt a highly folded conformation in water, most probably as a β-helix, and is monomeric. The metal ion-bound forms are clearly discernible in the monomers. The presence of dimeric form is not observed. It is unlikely this is due to the operating conditions or the method used, as both hetero- and homodimers in gramicidin D are detected when methanol is used as a solvent (Figure 5). ESI mass spectrometry studies suggest that gramicidin A retains a history of solvent environment in the gas phase.[27,28] Dimers have also been detected in the gas phase when the peptide is present as a dimer in solution. In the MALDI method, ions are generated from the sample, which is in the solid state. No dimers were detected when gramicidin A was dried from 2,2,2-trifluoroethanol solution, in which gramicidin A exists in the monomeric form.[3,4] The detection of dimers when the sample is dried from a methanolic solution suggests the presence of a population of noncovalent dimers in the solid state. The present study establishes that retention of solvent history is also observed when ions are generated by an entirely dif-

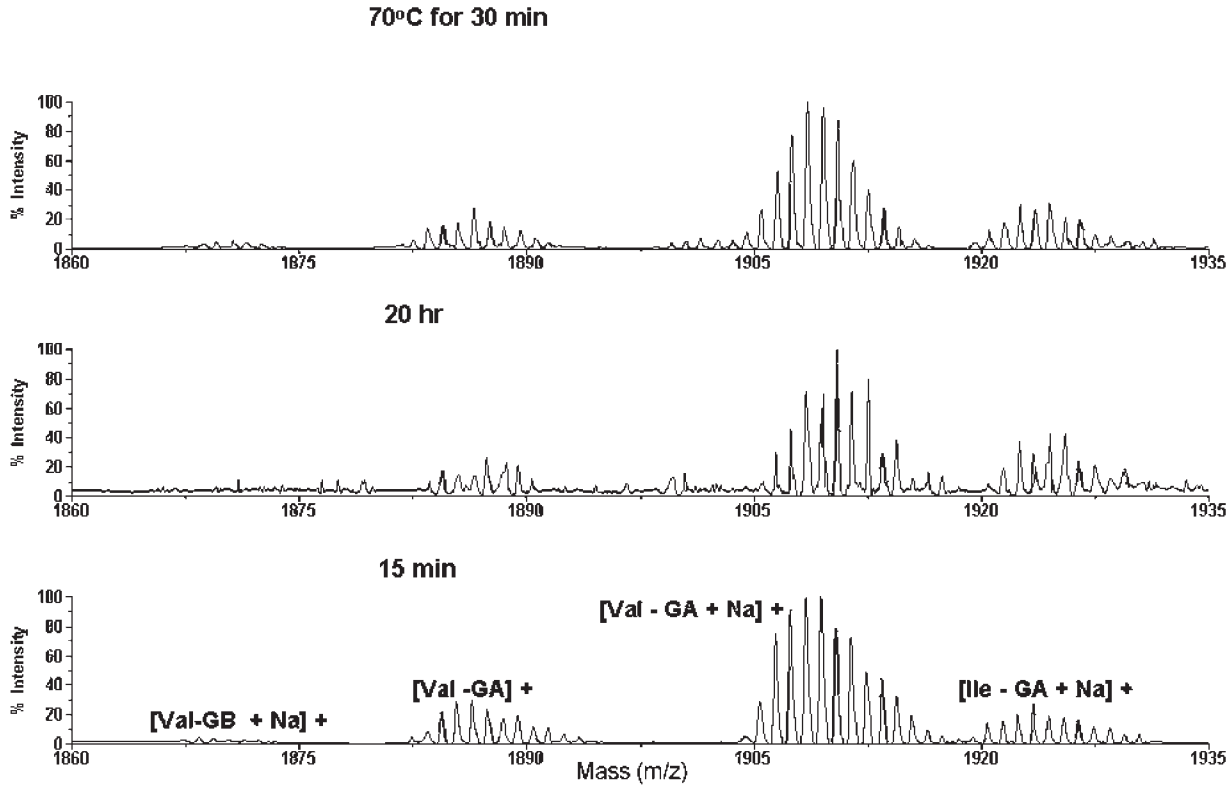

FIGURE 2 MALDI-TOF mass spectra of gramicidin D in D_2O using deuterated CHCA as matrix. Spectra were recorded 15 min and 20 h after exchange was initiated at 25°C. Spectrum shown in the top panel was obtained after gramicidin solution was heated at 70°C for 30 min in D_2O immediately after the exchange was initiated. The clusters show isotopic distribution.

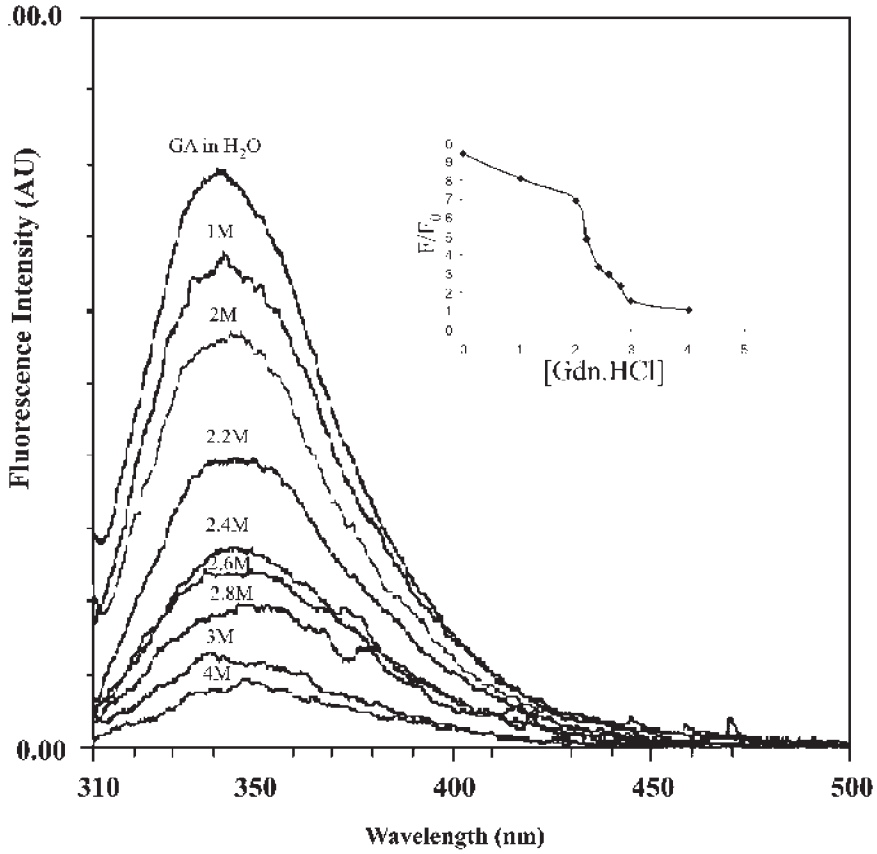

FIGURE 3 Emission spectra and (inset) changes in fluorescence intensity of gramicidin D (2 μM) as a function of guanidine hydrochloride (Gdn · HCl) concentration.

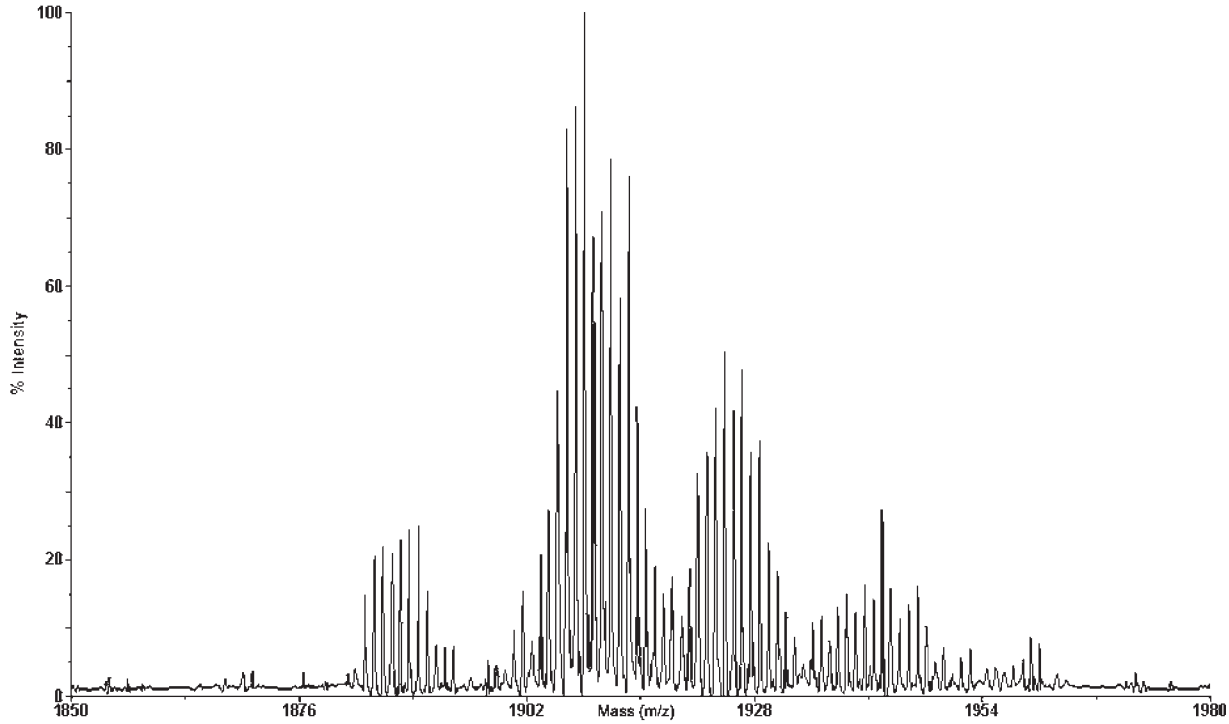

FIGURE 4 MALDI-TOF mass spectrum of gramicidin D after deuterium exchange in the presence of 4*M* deuterated denaturant. The clusters show isotopic distribution.

ferent process, i.e., MALDI and analyzed by TOF. Our study demonstrates the usefulness of MALDI-TOF mass spectrometry in studying the conformational behavior of neutral hydrophobic peptides that have very low solubility in water and in assessing their oligomeric status.

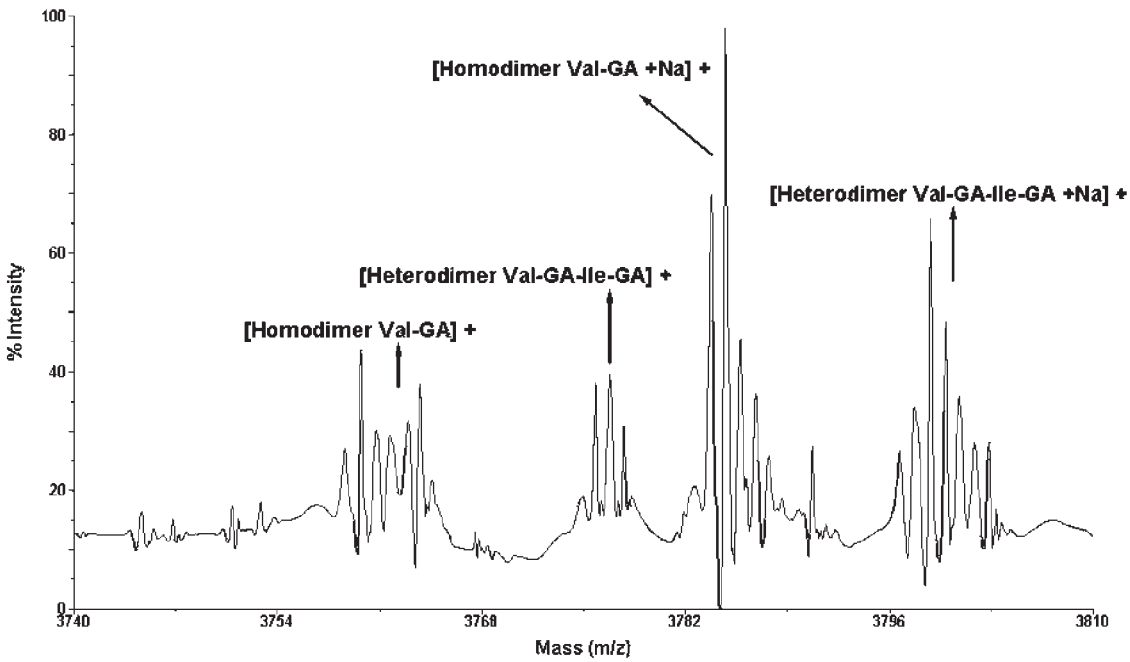

FIGURE 5 MALDI-TOF mass spectrum of gramicidin D from methanol solution in the *m*/*z* 3740–3810 range showing homo- and heterodimers.

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