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Thallous Ion Interaction with Gramicidin Incorporated in Micelles Studied by Thallium-205 Nuclear Magnetic Resonance†

J. F. Hinton,* G. Young, and F. S. Millett

ABSTRACT: A study has been made of the interaction between the thallous ion and gramicidin dimers incorporated into micelles by using ^{205}Tl NMR spectroscopy. The chemical shift data obtained are interpreted in terms of a model in which the dimer has only one tight binding site. The binding constant for this site was determined to be 900 M^{-1} at 30 °C. The thallous ion also appears to be partially solvated in the channel.

Thallium-205 NMR spectroscopy has been shown to be a very sensitive probe for studying the interaction between antibiotics and the thallous ion in solution (Hinton & Briggs, 1978; Briggs et al., 1980; Briggs & Hinton, 1978a,b) and in the solid state (Hinton et al., 1981a). Recently, we have investigated the complexation of the thallous ion by gramicidin A in dioxane (Hinton et al., 1981b) and in trifluoroethanol solutions (Turner et al., 1981) using ^{205}Tl NMR. Gramicidin A is a linear polypeptide antibiotic that has been very well characterized for its ability to form ion-selective membrane channels (Hladky & Haydon, 1970, 1972; Krause et al., 1971; Mueller & Rudin, 1967; Myers & Haydon, 1972; Neher, 1975; Sandblom et al., 1977). In membranes the channel is formed by gramicidin dimers (Tosteson et al., 1968; Goodall, 1970; Urry et al., 1971; Bamberg & Lauger, 1973; Zingsheim & Neher, 1974; Veatch et al., 1975; Veatch & Stryer, 1977) connected by N-terminal to N-terminal interaction (Goodall, 1971; Urry et al., 1971; Bamberg et al., 1977; Bamberg & Janko, 1977; Bradley et al., 1978; Szabo & Urry, 1979; Apell et al., 1977; Morrow et al., 1979; Weinstein et al., 1979, 1980).

It was of interest to study the interaction between the thallous ion and gramicidin incorporated into micelles by using the ^{205}Tl NMR technique for several reasons: (1) to compare the results with those obtained previously in trifluoroethanol and dioxane solutions; (2) to compare the NMR results with those obtained by equilibrium dialysis (Veatch & Durkin, 1980) and electrical measurements (Eisenman et al., 1978; Levitt, 1978; Hladky et al., 1979) where gramicidin was incorporated in a membrane; (3) to obtain the number of strong binding sites per dimer channel; (4) to determine the binding constant; (5) to try to determine the extent of hydration of the ion in the channel.

Materials and Methods

Gramicidin was incorporated into lysolecithin micelles by using the procedures of Urry (Urry et al., 1979; Spisni et al., 1979). L-α-Lysolecithin from egg yolk, containing primarily palmitic and stearic acids at position one, was obtained from Sigma Chemical Co., St. Louis, MO, and was used without further purification. Gramicidin was obtained from ICN and was a mixture composed of approximately 85% gramicidin A, 10% gramicidin B, and 5% gramicidin C. Thallium nitrate from Alfa was twice recrystallized before use.

Lysolecithin was weighed under N_2 atmosphere and suspended in 3 mL of degassed water. This suspension was sonicated in an ice bath under N_2 flow for 3 min with a Branson W-185 cell disruptor fitted with a microtip. Gramicidin was added under N_2 atmosphere to give a lysolecithin:gramicidin ratio of approximately 10:1. The suspension was shaken until all of the gramicidin was dispersed and then sonicated for 6 min in an ice bath under N_2 flow. Samples were then heated in a water bath, with agitation, for 15 h at 68 °C.

Samples for ^{205}Tl NMR spectroscopy were prepared by diluting aliquots of the lysolecithin–gramicidin suspension with 0.2 M $TlNO_3$ degassed aqueous solution to give the desired $TlNO_3$ and gramicidin concentrations. Samples were stored at 68 °C until placed in the spectrometer.

For each gramicidin-containing sample an aqueous $TlNO_3$ solution at the same Tl^+ concentration was prepared. The resonance frequency of this samples was used as the "free" ion resonance frequency for that $TlNO_3$ concentration in calculating the gramicidin induced shift.

† From the Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701. *Received July 13, 1981.* We wish to acknowledge the support of the National Science Foundation through Grant PCM-7827037.

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For correction for the effect of just the lysolecithin micelles on the $TlNO_3$ resonance frequency, a series of samples containing the same lysolecithin concentration as the gramicidin samples and with $TlNO_3$ concentration varying over the same range were prepared. The lysolecithin-induced shift in the ^{205}Tl resonance frequency at a given $TlNO_3$ concentration was subtracted from the gramicidin-induced shift to remove the lysolecithin effect. The assumption is made that the incorporation of gramicidin into the micelle does not significantly alter the properties of the micelle toward the binding of the thallous ion. The correction for micelle binding was most significant in the high thallous ion concentration range. It was found that the lysolecithin micelles induced a shift to high frequency in the Tl-205 resonance frequency while the gramicidin-induced shift was to low frequency referenced to an aqueous solution of $TlNO_3$ at the same salt concentration.

The pertinent data obtained were, therefore, the gramicidin-induced shift of the thallous ion resonance frequency for a fixed gramicidin concentration as a function of varying thallous ion concentration.

All spectra were obtained with a Bruker HFX-90 spectrometer modified for pulsed Fourier transform operation. The probe temperature was controlled at 30 °C.

For Tl^+ ions rapidly exchanging between a free state (f) in solution and a gramicidin bound state (b), the observed chemical shift, δ_0, is given by

$$\delta_0 = \chi_f\delta_f + \chi_b\delta_b \tag{1}$$

where χ_f, χ_b, δ_f, and δ_b are the mole fractions and chemical shifts of the free and bound state, respectively. If we let $\delta_f = 0$ (i.e., the free state chemical shift is used as the reference), then

$$\delta_0 = \chi_b\delta_b \tag{2}$$

Model I. If, in considering the Tl^+–gramicidin interaction, we assume a single tight binding site per dimer, one may derive the following equation for the observed chemical shift.

$$Tl^+ + Gr_2 \rightleftarrows Tl^+Gr_2 \tag{3}$$

Let $A = Tl^+$, $E = Gr_2$, and $\chi = Tl^+Gr_2$ so that

$$A + E \rightleftarrows X \tag{4}$$

and the equilibrium constant is then

$$K = \frac{X}{AE} \tag{5}$$

Therefore

$$\chi_b = \frac{X}{A_0} \tag{6}$$

where A_0 = total $[Tl^+]$. Therefore

$$\delta_0 = \left(\frac{X}{A_0}\right)\delta_b \tag{7}$$

or

$$\chi = A_0(\delta_0/\delta_b) \tag{8}$$

Now since

$$A = A_0 - X = A_0 - \left(\frac{\delta_0}{\delta_b}\right)A_0 \tag{9}$$

$$E = E_0 - X = E_0 - (\delta_0/\delta_b)A_0 \tag{10}$$

the equilibrium constant is then

$$K = \frac{(\delta_0/\delta_b)A_0}{[A_0 - (\delta_0/\delta_b)A_0][E_0 - (\delta_0/\delta_b)A_0]} \tag{11}$$

or solving for the observed chemical shift

$$\delta_0 = \frac{(KA_0 + KE_0 + 1) - \sqrt{(KA_0 + KE_0 + 1)^2 - 4(KA_0)(KE_0)}}{2(KA_0/\delta_b)} \tag{12}$$

For the case in which there are two available tight binding sites per dimer, but the occupation of one forbids the occupation of the other, the mathematical treatment will be identical with the above except that K is replaced by $2K_s$, where K_s is the site binding constant.

Model II. Finally, if we consider the case of simultaneously occupiable, and noninteracting identical sites per dimer, we have

$$Tl^+ + Gr \rightleftarrows Tl^+Gr \tag{13}$$

where Gr represents the total gramicidin concentration (i.e., the monomer concentration). The observed chemical shift is then

$$\delta_0 = \frac{(KA_0 + KE_0 + 1) - \sqrt{(KA_0 + KE_0 + 1)^2 - 4(KA_0)(KE_0)}}{2(KA_0/\delta_b)} \tag{14}$$

The method of Duggleby (1981) was used to fit the experimental data (i.e., the observed chemical shift, δ_0, as a function of the thallous ion concentration) for all three models.

Results

The model found to be most consistent with the experimental data was model I. Figure 1 shows the fit of the theoretically determined relationship between chemical shift and thallous ion concentration to the experimental data. This result is consistent with that obtained by equilibrium dialysis (Veatch & Durkin, 1980) which indicated a maximum of only one occupied highest affinity binding site per channel. The equilibrium dialysis experiments (Veatch & Durkin, 1980) suggested that the concentration at which the second Tl^+ ion binds to the channel must be 20 mM or higher. The ^{205}Tl NMR results were obtained for a Tl^+ ion concentration range of about 2.5–65 mM, and the analysis of this data does not indicate a second binding site at the higher Tl^+ ion concentrations, although a second site with a *very* small binding constant might be present but not be detectable by the chemical shift method.

The binding or equilibrium constant obtained from the mathematical analysis is $K_s = 900\ M^{-1}$ at 30 °C. This value seems consistent with the value of 500–1000 M^{-1} obtained in the equilibrium dialysis experiment at 23 °C (Veatch & Durkin, 1980) and electrical measurements which gave 550 (Eisenman et al., 1978), about 500 (Levitt, 1978), and 9000 M^{-1} (Hladky et al., 1979). A value of $K = 566\ M^{-1}$ at 25 °C was obtained from trifluoroethanol solution (Turner et al., 1981) where both ends of the gramicidin dimer were found to bind to the Tl^+ ion.

It seems clear from the value of the complex shift, δ_b, obtained that the Tl^+ ion is still appreciably solvated by water molecules. The resonance frequency of the free Tl^+ ion in pure water is 51 915 157 Hz, that of the Tl^+Gr_2 complex for the micelle solution is 51 911 788 Hz, and that of thallium in the intact Tl^+Gr_2 complex in dioxane is 51 880 400 Hz (Hinton et al., 1981a).

There is one aspect of the analysis of the data that appears to indicate that the analytical concentration of the gramicidin dimer is not the effective channel dimer concentration. The

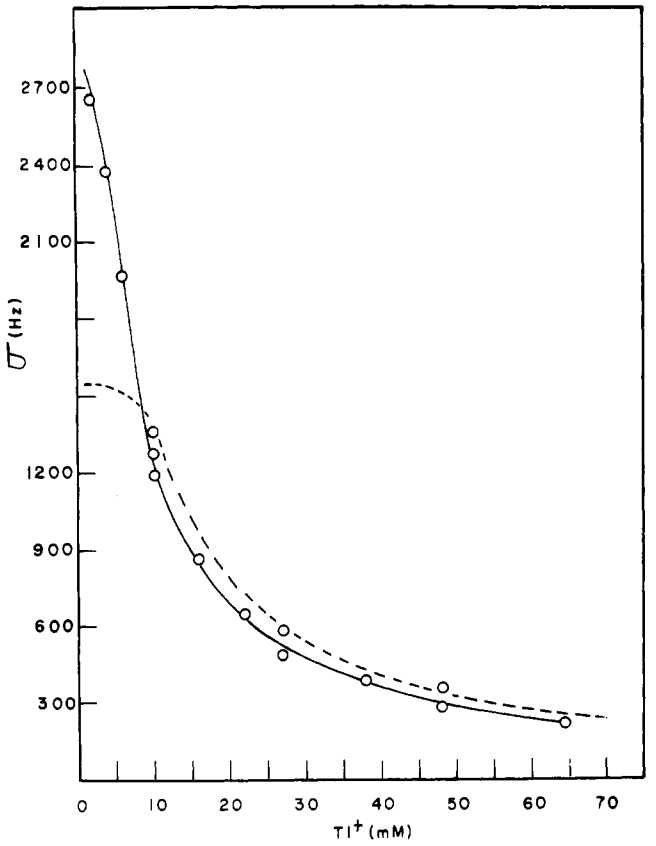

FIGURE 1: Corrected ^{205}Tl chemical shift plotted as a function of the thallous ion concentration (open circles). The solid line is a theoretical representation of the shift–concentration relationship for model I. The dashed line is a theoretical representation of the shift–concentration relationship for model II. The temperature is 30 °C.

analytical concentration of dimers was 0.00515 M; however, it was found that a fit to the experimental data was better when a value of 0.0045 M was used. This seems to be consistent with what is known about the mobility of gramicidin in micelles and vesicles and the dimer lifetime in such model membrane systems.

For illustration of the point that model II does not appear to fit the experimental data, a "best-fit" analysis of this model is shown along with the experimental points in Figure 1. Here we see that the fit is very poor. The complex shift, δ_b, is found to be much lower than the low concentration experimental points, and the breakoff in the theoretical curve at low Tl^+ ion concentrations is indicative of a poor fit (see Appendix for a discussion of why the breakoff in the curve comes lower than δ_b).

In summary, the ^{205}Tl NMR data indicate one tight binding site for gramicidin dimers incorporated into micelles whose site binding constant at 30 °C is about 900 M^{-1}. The Tl^+ ion appears to be highly solvated at the binding site.

Appendix

If, using model I, the observed shift is given by

$$\delta_0 = \frac{KA_0 + KE_0 + 1 - \sqrt{(KA_0 + KE_0 + 1)^2 - 4K^2A_0E_0}}{2KA_0/\delta_b}$$

the y intercept of a graph of δ_0 vs. $Tl^+(A_0)$ will not be the bound shift δ_b but will be

$$\lim_{A_0 \to 0} \delta_0 = \lim_{A_0 \to 0} \frac{KA_0 + KE_0 + 1 - \sqrt{(KA_0 + KE_0 + 1)^2 - 4K^2A_0E_0}}{2KA_0/\delta_b}$$

Direct substitution of $A_0 = 0$ gives

$$\lim_{A_0 \to 0} \delta_0 = \frac{KE_0 + 1 - \sqrt{(KE_0 + 1)^2 - 0}}{0} = \frac{0}{0}$$

For the quotient of two functions $f(x)/g(x)$, where $\lim_{x \to c} f(x)/g(x) = 0/0$, if the limit of the quotient of the derivatives of the function is m

$$\lim_{x \to c} f'(x)/g'(x) = m$$

Then

$$\lim_{x \to c} f(x)/g(x) = m$$

Using $f(A_0) = KA_0 + KE_0 + 1 - [(KA_0 + KE_0 + 1)^2 - 4K^2A_0E_0]^{1/2}$ and $g(A_0) = 2KA_0/\delta_b$, we obtain

$$\lim_{A_0 \to 0} \frac{f'(A_0)}{g'(A_0)} = \left[\frac{1 - [(1 - KE_0)/(1 + KE_0)]}{2}\right]\delta_b = \left(\frac{KE_0}{1 + KE_0}\right)\delta_b$$

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Steroid–Protein Interactions. Human Corticosteroid-Binding Globulin: Characterization of Dimer and Electrophoretic Variants†

Kenneth E. Mickelson,*,‡ George B. Harding,§ Michael Forsthoefel,∥ and Ulrich Westphal

ABSTRACT: Human corticosteroid-binding globulin (CBG) forms a dimer that was isolated by gel filtration, has full binding affinity and capacity, and can be dissociated to the monomer. Monomeric CBG consists of two distinct molecular variants, which were detected by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. The two monomeric CBG species were separated by preparative gel electrophoresis and were found to bind cortisol, as well as progesterone, with equal affinity. They have one steroid binding site per CBG molecule. Amino acid and carbohydrate analyses are essentially the same for both of the CBG variants. Removal of sialic acid or 90% of the carbohydrate did not affect the existence of the two molecular forms. The two CBG species were isolated from each of the sera from five individual donors, indicating that the observed heterogeneity does not result from pooling genetic variants. The two species are immunologically identical. A possible explanation for the existence of the two electrophoretic variants is a difference in amidation.

More than 20 years have past since the first reports on the corticosteroid-binding globulin (CBG)[1] of human blood serum were published (Daughaday, 1956; Bush, 1957; Sandberg & Slaunwhite, 1958). CBG has been extensively studied in the last 5 years since the protein became more readily available following the use of affinity chromatography for its isolation (Rosner & Bradlow, 1971, 1975; Trapp et al., 1971; Le Gaillard et al., 1974). CBG consists of one single polypeptide chain containing about 30% carbohydrate and having a molecular weight of about 52 000 (Westphal, 1971; Le Gaillard et al., 1975). One molecule of CBG binds one molecule of cortisol or progesterone with high affinity ($K_a \sim 10^9$ M^{-1}, 4 °C). Recently, human CBG was reported to form a dimer with full binding activity (Stroupe et al., 1977) and to consist of two molecular variants separable by electrophoresis in acrylamide gels of low porosity (Harding et al., 1978a,b). This paper reports the results of binding and compositional analyses of the various species of human CBG.

Materials and Methods

Term human pregnancy serum was obtained from Louisville's Norton-Children's Hospital (residual samples from routine testing) and University Hospital (placental blood). Outdated human blood was obtained from the American Red Cross (Louisville, KY). Amberlite XAD-2 and XAD-4 was from Rohm and Haas; it was washed 10 times with equal volumes of methanol and then in a similar way with distilled water at room temperature. Radiolabeled steroids were purchased from New England Nuclear. The radiopurity was checked by thin-layer chromatography. Endogenous steroids were removed by gently shaking 1 L of serum with 200 mL (settled volume) of Amberlite XAD-2 resin for 8 h at room temperature. The serum was then filtered through a fritted disk funnel (coarse), dialyzed for 40 h against 50 mM phosphate–0.5 M KCl (pH 9.0, 4 °C, 0.02% sodium azide), centrifuged for 30 min at 12000*g*, and filtered through a Whatman no. 2 filter paper.

Purification of human CBG was achieved by affinity chromatography with agarose to which 11β-hydroxy-3-oxo-4-androstene-17β-carboxylic acid was coupled through an

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‡ Present address: Miles Laboratories, Inc., Elkhart, IN 46515.
§ Present address: Department of Medicine, University of Louisville, Louisville, KY 40292.
∥ Present address: Emory University, Atlanta, GA 30322.

[1] Abbreviations: CBG, corticosteroid-binding globulin; K_a, equilibrium association constant; n, number of binding sites per molecule of protein; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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