The Effect of Aggregation on the Binding of a Derivative of the Glycopeptide Antibiotic Teicoplanin to a Model Tripeptide

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Much attention is being devoted to glycopeptide antibiotics because of both their therapeutic relevance[1] and the wealth of information they are giving on fundamental aspects of molecular recognition.[2] Their mode of action involves the binding of peptides of the bacterial cell walls showing the C-terminal sequence L-X-D-Ala-D-Ala (where X = lysine, alanine, homoserine, or diaminopimelic acid). Peptide models showing high affinity for this class of antibiotics have been designed in order to study the thermodynamic and kinetic features of the binding process. Among them the most studied are α,ε-*N*,*N*'-diacetyl-L-Lys-D-Ala-D-Ala (AALAA) and ε-*N*-acetyl-α-*N*-dansyl-L-Lys-D-Ala-D-Ala (ADLAA). The latter one has been recently introduced by Pratt and his associates[3] and proved particularly useful for the determination of large binding constants ($K_b > 10^6$ M^{-1}) and kinetic parameters related to the binding process.[4].

The thermodynamics of binding appear to be governed by hydrophobic and polar (hydrogen bonding, charge–charge) interactions.[2] NMR experiments proved very useful to define the points of interaction between the antibiotic and the peptide:[5] in the case of vancomycin five hydrogen bonds govern the geometry of binding.

The kinetics of binding show that the rates are much slower than expected for a diffusion-controlled bimolecular process.[4] For this reason, Pratt[4b] has recently proposed a two-step mechanism involving a fast preequilibrium followed by a slower evolution to the final complex. This slow process appears to involve rearrangement of the solvent with hydrogen bonds breaking.

Another aspect that characterizes at least some of these antibiotics is their tendency to aggregate. Williams[6] and others[7] have recently shown that dimerization may be quite relevant (for instance, eremomycin has $K_{dim} \geq 10^5$ M^{-1} and vancomycin has $K_{dim} = 700$ M^{-1}) while, previously, less defined aggregation phenomena have been described for vancomycin[8] and teicoplanin.[9] It has been suggested that the formation of these aggregates may influence the action of these antibiotics.[6]

In this paper we report evidence that a semisynthetic derivative of teicoplanin,[10] where the primary amine of the side chain has been simply protected by conversion into the benzylcarbamate, CTA-Cbz, Figure 1, shows amphiphilic properties leading to the formation of aggregates and how aggregation influences the lipophilicity of the binding site and the rate of the association to the model peptide ADLAA.

Results and Discussion

The derivative CTA-Cbz has already been described:[11] it is prepared by reacting benzyl chloroformate with teicoplanin A2 (CTA). Its structure is illustrated in Figure 1.

Previous work[9] reported that at concentrations $>2 \times 10^{-4}$ M teicoplanin forms aggregates; this accounts for higher retentions of antibiotic, observed above this concentration, in affinity chromatography. We observed that this tendency of the antibiotic to aggregate is also associated with a surfactant-like behavior. When the surface tension was measured for aqueous solutions of teicoplanin a decrease was observed as the concentration of the antibiotic increased and the plot of surface tension *vs* concentration roughly defined two linear tracts intersecting at [teicoplanin] = 2×10^{-4} M (Figure 2, curve a). It is the typical behavior of micelle-forming surfactants and the concentration at the intersection point is taken as the critical micelle concentration (cmc), *i.e.*, the onset of micellization.[12] In the present case, since the type and shape of the aggregates are not defined, it will be termed critical aggregate concentration (cac).[13] The changes of surface tension are also associated with an increase of the intensity of the scattered light suggesting the formation of aggregates of larger size than monomeric teicoplanin for concentration exceeding 2.1×10^{-4} M. The derivative CTA-Cbz, which is less hydrophilic than its parent compound, also shows a surfactant-like behavior and decreases the surface tension; in this case the cac

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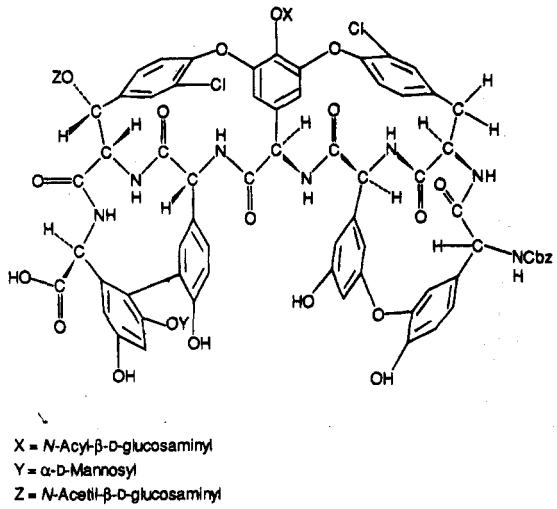

Figure 1. Structure of the semisynthetic derivative of teicoplanin, CTA-Cbz.

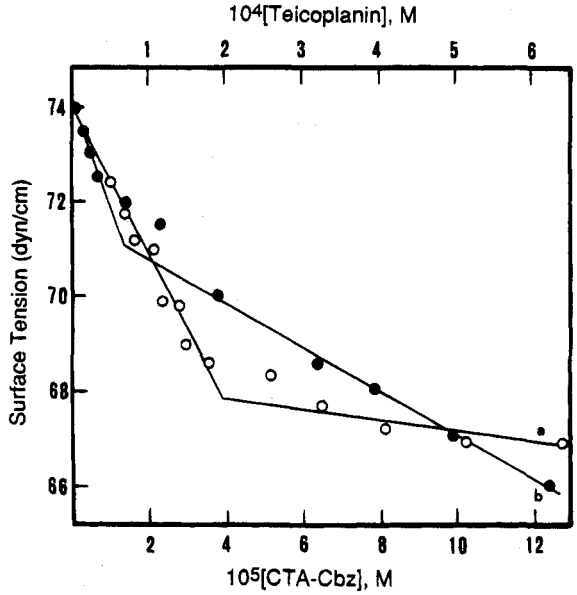

Figure 2. Surface tension against antibiotic concentration as determined in 0.1 M phosphate buffer (pH = 7.0) at 25.0 °C: (a, ○) teicoplanin; (b, ●) CTA-Cbz.

value, ~1.4 × 10^{-5} M (see Figure 2, curve b), is lower than that of the parent antibiotic. At concentrations higher than the cac, the surface tension of solutions of CTA-Cbz continues to decrease suggesting that the aggregates formed have not reached a stable conformation and are still surface tension active.[14] The low value of the cac (20 times smaller than that of teicoplanin) as well as the small dimensions of the aggregates of this system did not allow us to monitor the onset of the CTA-Cbz aggregates by following the change of the intensity of the scattered light: this falls well within the noise of the background.

When the affinity constant of CTA-Cbz for the model peptide ADLAA was determined by following the increase in the fluorescence intensity of the peptide while increasing the antibiotic concentration, we observed the profile

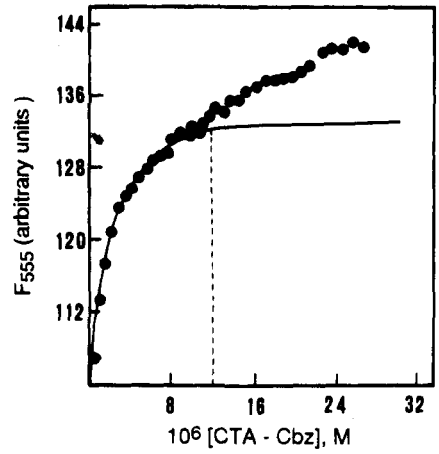

Figure 3. Change of fluorescence intensity of ADLAA (0.5 μM) at 555 nm as a function of CTA-Cbz concentration at 25.0 ± 0.1 °C. The solid line represents the computer calculate curve for $K_b = 1.5 \times 10^6$ M^{-1}. The vertical dotted line indicates the cac value obtained from the surface tension measurement.

shown in Figure 3. Up to a concentration close to 1.0 × 10^{-5} M the increase of fluorescence intensity follows a normal pattern giving the binding constant $K_b = 1.5 \times 10^6$ M^{-1} as determined by nonlinear regression analysis (see Experimental Section). However, above this concentration, instead of flattening, having reached the fluorescence of the fully-bound peptide, the fluorescence intensity further increases. The concentration at which we observe the onset of this phenomenon is very close to the cac determined tensiometrically. This change of fluorescence intensity reflects a change of the environment, from a more hydrated to a less hydrated region, experienced by the dansyl moiety of the bound tripeptide, as a consequence of the aggregation of the antibiotic.[15] The above evidences (i) the fact that the value of the cac decreases by almost 1 order of magnitude in CTA-Cbz with respect to teicoplanin as the result of an added hydrophobic substituent and (ii) the increase in fluorescence intensity of the complex with ADLAA in aggregates suggests a major contribution of hydrophobic interactions in triggering the aggregation process under study. Williams[6] has clearly shown how dimerization of some of these antibiotics is an important process driven by polar interactions involving charged ammonium groups in the sugar residues, hydrogen bonding of the peptide backbone, and solvent exclusion. Though all these interactions may contribute to some extent, it is apparent the major role is played by the hydrophobic effect in the present aggregation process.

Following Pratt's[4b] suggestion that the slow kinetic step in the binding process involves the breaking of hydrogen bonds of solvent molecules, it was interesting to determine if the rate of binding could be influenced by the aggregation. Accordingly, stopped-flow experiments analogous to those described by Pratt were carried out with CTA-Cbz. The results, reported in Figure 4, show a clear change of slope in the plot k_{obs} *vs* [CTA-Cbz] at the antibiotic concentration ~1.0 × 10^{-5} M, again close to the cac evaluated from the surface tension and fluorescence measurements described above. At higher concentration the dependence of the k_{obs} from [CTA-Cbz] is less pronounced, the slope of the second part of the

(14) As a consequence, when [CTA-Cbz] is increased the number of small aggregates would increase and, hence, the surface tension decreases. We thank a reviewer for pointing out this aspect of aggregation to us.

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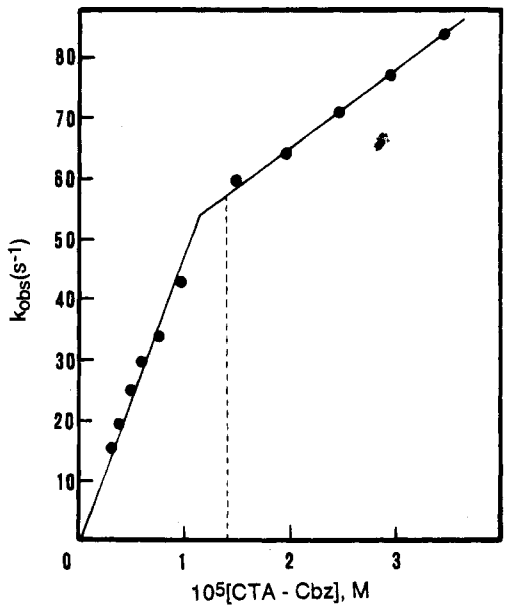

Figure 4. Variation of the pseudo-first-order rate constant at 25 °C of association of ADLAA (2 μM) with CTA-Cbz as a function of CTA-Cbz concentration. The solid line represents the linear least-squares fit to the data. The vertical dotted line indicates the cac value obtained from the surface tension measurement.

plot being *ca.* 3.2 times smaller than that of the first one. Following the kinetic treatment given by Pratt,[4b] assuming a two-step process

$$A + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AP^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AP$$

kinetically controlled by the second rate-limiting step, where A is the antibiotic, P the peptide, and AP* and AP the precomplex and final complex, respectively. The dependence of the observed rate constant, k_{obs}, from the concentration of antibiotic (under conditions $[A]_o \gg [P]_o$ and assuming $K_1^{-1} \gg [A]_o$) should follow eq 1:

$$k_{obs} = k_2 K_1 [A]_o + k_{-2} \quad (1)$$

Accordingly, a change in the slope of the plot k_{obs} *vs* $[A]_o$ at $[A]_o$ = cac may reflect a change in (any or all) the following parameters: (i) K_1, the precomplex formation constant; (ii) the concentration of antibiotic "available" for binding; and (iii) the rate constant k_2.

Assuming that the product k_2K_1 is not greatly affected by the aggregation status of the antibiotic, the observed rate constant could depend more closely on the aggregate concentration[16] rather than on the stoichiometric antibiotic concentration.[17] However, even considering that, in the range of concentrations explored, we are just above the cac and the average aggregation number is small, one can hardly assume that K_1 and k_2 do not change and so this fact must be considered, too. Although a decrease of the diffusion coefficient[18] (and hence k_1) can be predicted, electrostatic and hydrophobic interactions would result in an increase rather than a decrease of K_1. On the other hand, a decrease of k_2 appears reasonable as the result of a higher desolvation demand inside rather than outside the aggregates.[19] This is suggested by the results of the binding experiments (see above) showing that ADLAA experiences a less hydrated environment when bound to the antibiotic as an aggregate. A major role of the desolvation in the formation of the final complex would further support the explanation given by Pratt[4b] to account for the slow rates of binding observed for some glycopeptide antibiotics.

Admittedly, in the absence of further information on the actual nature of the aggregates there is little room for further speculation.

The derivative CTA-Cbz is characterized by a quite fortunate value of the cac. A too high cac, in fact, hampers the determination of the rate constants which become too fast (this is the case of teicoplanin), while a too low cac becomes difficult to be detected. This would be the case of less hydrophilic derivatives of the glycopeptide antibiotics. For these molecules, since in water aggregation would occur even at very low concentration, there is no way but to study the system as an aggregate rather than an isolated molecule. Since, as pointed out already by Williams,[6] quite likely aggregation plays a role in governing the activity of these antibiotics, it is clearly important to ascertain the aggregation status of the glycopeptide derivative when relating the binding with model peptides to their biological activity.

Experimental Section

Instrumentation. Surface tension measurements were performed using a Krüss type 8451 tensiometer. Light scattering determinations were performed with a Nicomp 370 autocorrelator equipped with a Spectra-Physics 2016 argon laser. Fluorescence spectra were recorded on a Perkin-Elmer MPF-66 spectrofluorimeter. Kinetics were followed on an Applied Photophysics SF. 17MV stopped-flow spectrometer.

Materials. Teicoplanin (component A2, CTA) and CTA-Cbz were obtained from Dr. A. Malabarba of the Lepetit Research Center. ADLAA was purchased from Backem (Switzerland) and used as received. Milli-Q water was used for the preparation of all solutions. Buffer solutions were prepared immediately before their use to avoid the formation of biological contaminants.

Equilibrium and Rate Constant Determination. Antibiotic stock solutions were prepared by suspending the proper amount of material in 5 mL of 0.1 M (pH = 7.0) phosphate buffer. The solution was sonicated for 15 min in a bath sonicator and filtered through a 0.22 μm Millipore filter and the concentration of the solution checked by measuring the absorbance at 280 nm at pH = 1 (HCl buffer). ADLAA solutions in 0.1 M phosphate buffer (pH = 7.0) were prepared by proper dilution of a 2.0×10^{-4} M stock solution. Fluorescence intensity was measured at 555 nm with an excitation wavelength of 330 nm in samples thermostated at 25.0 ± 0.1 °C; $[ADLAA] = 5.0 \times 10^{-7}$ M. The

(16) The concentration of aggregates C_A can be obtained from the following equation: $C_A = ([A]_o - \text{cac})/\text{aggregation number}$. The aggregation number, *i.e.*, the number of antibiotic units forming the aggregate, should roughly be in the 3–5 range. This is in accord with the very low intensity of scattered light observed for these aggregates.

(17) This would imply that the precomplex formation in aggregates depends on a diffusion-controlled collision between the peptide and a single antibiotic. If any intraaggregate collision is allowed one must take into account that the "available" antibiotic concentration is larger than that of the aggregate and could even exceed $[A]_o$ (this usually happens in functional-micelle catalysis; see, for instance: Bunton, C. A.; Savelli, G. *Adv. Phys. Org. Chem.* **1986**, *22*, 213).

(18) We assume a 15 Å radius for these aggregates close to that found for small micelles (see ref 12, pp 30–31).

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binding isotherm was fitted using the program HOSTEST II.[20] The fitting was limited to the concentration interval before the cac. Kinetics were followed by monitoring the change with time of the fluorescence intensity at $\lambda > 420$ nm (using a proper filter) with excitation $\lambda = 330$ nm upon mixing antibiotic and ADLAA solutions, [ADLAA] = 2.0×10^{-6} M. Curve fitting was performed by nonlinear regression analysis using the software program provided with the stopped-flow instrument.

Acknowledgment. The authors are indebted to Dr. A. Malabarba for providing the samples of CTA-Cbz and teicoplanin, Prof. C. S. Wilcox for the program HOSTEST II, and Mr. E. Castiglione for technical assistance.

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