Oxygen Binding and pH Stability of Tubular Polymers from *Helix pomatia* β_c-Hemocyanin†

Jan M. van der Laan,‡ Ruurd Torensma,* and Ernst F. J. van Bruggen

ABSTRACT: Limited trypsinolysis of native cylindrical *Helix pomatia* β_c-hemocyanin removes the collar. The resulting hollow cylindrical molecules polymerize end to end in long tubular polymers. Oxygen-binding behavior of these polymers is highly cooperative and largely affected by the pH. The oxygen-binding curves were analyzed in the framework of a two-state model. Oxygen-binding parameters obtained from the analysis indicate that interactions between the oxygen-binding domains are not influenced by the association into tubular polymers. Only the interaction free energy per site is increased. The results suggest interacting oxygen-binding domains are a set of nearest neighbors having the correct orientations toward each other. The number of interacting sites is dependent on pH and Ca^{2+} ions. The dissociation of tubular polymers as influenced by pH, ionic strength, and calcium ion concentration was determined. With an increase in the pH, in the absence of calcium ions, tubular polymers first dissociate into smaller tubular polymers, followed by a complete dissociation into a mixture of multidomain fragments. The latter dissociation is hardly dependent on ionic strength. In the presence of calcium ions, the pH span, where dissociation occurs, is greater. Dissociation products resembling half molecules are observed. Their occurrence is highly dependent on ionic strength.

Hemocyanins are large copper-containing proteins. They are responsible for oxygen transport in many arthropods and molluscs and occur freely dissolved in the hemolymph. The active site contains two copper atoms that bind one oxygen molecule. The hemocyanin of the Roman snail *Helix pomatia* (M_r 9 000 000) is not homogeneous. It contains 75% α component, which dissociates in 1 M NaCl at pH 5.7; the remaining 25%, which does not dissociate, is called β component (Heirwegh et al., 1961). The latter is constituted of two fractions, one precipitating at pH 5.3 ($\beta_{crystalline}$, β_c) and one that does not ($\beta_{soluble}$, β_s) (Gielens et al., 1975; Préaux et al., 1979; Kuiper et al., 1976).

β_s-Hemocyanin and α-hemocyanin have the same electrophoretic mobility at pH 8.6 and are immunological identical; β_c-hemocyanin, on the contrary, has a lower electrophoretic mobility and differs in some of its antigenic sites (Gielens et al., 1973, 1979; Préaux et al., 1981). The shape of gastropod hemocyanins is a hollow cylinder partly closed at both ends by a collar and a cap (Mellema & Klug, 1972). Increasing the pH and removing divalent cations like Ca^{2+} and Mg^{2+} cause dissociation into subunits, viz., half, tenth, and twentieth molecules (Siezen & Van Driel, 1974). For β_c-hemocyanin, the cylindrical structure is formed by the assembly of 20 identical polypeptide chains, each folded into eight oxygen-binding domains (Lontie & Gielens, 1979). Per polypeptide chain, six domains belong to the wall of the cylinder (Van Breemen et al., 1977).

Binding of oxygen by the native cylindrical molecule proceeds cooperatively with a maximum Hill coefficient of 7 at pH 7.18 and with a negative Bohr effect (Zolla et al., 1978). The number of interacting sites was found to be pH dependent. Below pH 7.4, the number of interacting sites is 15, decreasing to eight above this pH. These numbers suggest that below pH 7.4 a functional constellation is equivalent to a tenth molecule and above this pH to a twentieth molecule (Zolla et al., 1978).

Limited proteolysis of native, as well as tenth, molecules results in a variety of domain mixtures (Gielens et al., 1975; van der Laan et al., 1981a; Gielens et al., 1981). Separation of these mixtures into virtually pure domains made it possible to demonstrate functional and structural differences between the eight domains of the polypeptide chain (van der Laan et al., 1981a; Torensma et al., 1980; Torensma et al., 1981b). Domains originating from the wall of the cylindrical molecule all bind oxygen noncooperatively, four having almost equal oxygen affinities (P_{50}[1] ~5 mmHg), one having low oxygen affinity (P_{50} ~14 mmHg), and one having high oxygen affinity (P_{50} ~2 mmHg). Only the latter displays a negative Bohr effect (Torensma et al., 1980). Limited proteolysis of native cylindrical molecules removes the collar, leaving hollow cylinders that polymerize in an end-to-end geometry leading to a heterogeneous population of tubular polymers (Van Breemen et al., 1975; Wood, 1977; Gullick et al., 1979). Besides the proteolytic cleavage of the polypeptide bond between the wall and the collar domains, several peptide bonds are cleaved between wall domains (van der Laan et al., 1981a). Cooperative oxygen binding is believed to be linked to the cylindrical shape of the molecule (Van Driel, 1973; Pearson & Wood, 1974). Therefore, the tubular portion of the native protein free of collar domains still should be capable to display cooperative oxygen binding behavior. We decided to study the oxygen-binding behavior to test if tubes are capable of binding oxygen cooperatively although part of the polypeptide had been removed. Moreover, we analyzed the effects of a changed structure on the oxygen-binding parameters. The dissociation behavior of the modified protein was investigated to relate possible functional properties with structural changes.

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[1] Abbreviations: P_{O_2}, oxygen pressure; P_{50}, oxygen pressure at half-saturation; Y, fractional saturation with oxygen; S, Svedberg; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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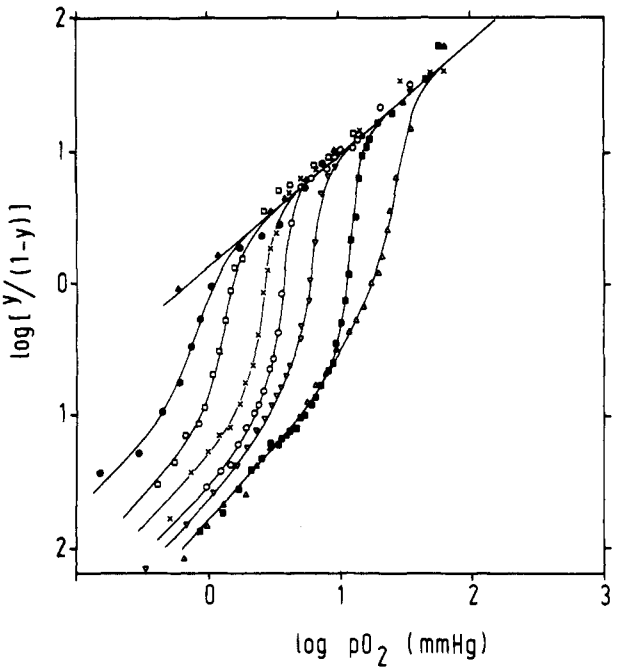

FIGURE 1: Hill plots of oxygen equilibrium curves of tubular polymers derived from *H. pomatia* β_c-hemocyanin. Conditions: pH 6–7, 20 mM Bistris-HCl; pH 7–8, 20 mM Tris-HCl to which 17 mM $CaCl_2$ was added. (▲) pH 6.55, (●) pH 6.88, (□) pH 7.05, (×) pH 7.20, (○) pH 7.27, (▽) pH 7.39, (■) pH 7.48, and (△) pH 7.80. Y is fractional saturation with oxygen; P_{O_2} is oxygen pressure (in mmHg). The continuous lines have been computed according to a two-state model, as described in the text.

Experimental Procedures

Hemocyanin from the Roman snail *Helix pomatia* was isolated according to Heirwegh et al. (1961). β_c-Hemocyanin was prepared from total hemocyanin as described earlier (Préaux et al., 1979; Kuiper et al., 1976). Buffer solutions were prepared according to Bates (1973). In the pH range 6–7, Bistris-HCl was used; in the range 7–9, Tris-HCl was used; and in the range 9–10.5, ethanolamine–HCl was used. Determination of hemocyanin concentration, analytical ultracentrifugation, and determination of oxygen-binding parameters were performed as previously described (Konings et al., 1969).

Tubular polymers were prepared by the method of Van Breemen et al. (1975) as modified by van der Laan et al. (1981a). The concentration of the tubular polymers was determined at pH 9.2 by measuring the absorbance at 278 nm, by using the same extinction coefficient as had been determined for β-hemocyanin (Heirwegh et al., 1961). For the oxygen-binding experiments, hemocyanin was regenerated with hydroxylamine according to Lontie & Witters (1966). Oxygen-binding curves were fitted with the theory developed by Monod et al. (1965). The best parameters describing the oxygen-binding behavior were calculated from a search program developed for the refinement of atomic parameters of heavy atoms (Hart, 1961). This program minimizes

$$\Sigma\left[\left(\frac{y}{1-y}\right)_{\text{calcd}} - \left(\frac{y}{1-y}\right)_{\text{exptl}}\right]^2$$

The use of the fitting program was very effective in obtaining the oxygen-binding parameters. Extracting even better values for these parameters now depends largely on better experimental data sets. Minimizing of the above function yields far more accurate values than comparing plotted fitted curves with the experimental data points.

To circumvent false minima due to inaccurate starting values (Blundell & Johnson, 1976), starting parameters were determined by means of a rough fit with the experimental P_{50}, k_T, and k_R and varying the number of interacting sites as integers. Calculations were performed on a CDC Cyber 175-760 computer. Electron micrographs were taken as described earlier (Van Breemen et al., 1975).

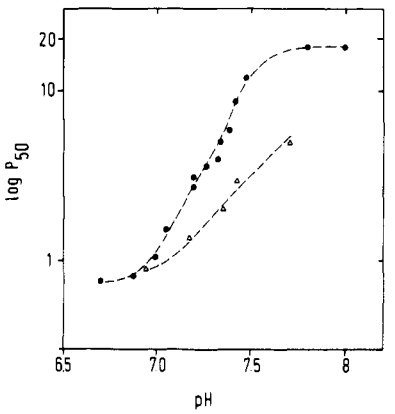

FIGURE 2: Plot of P_{50} values as a function of pH in the absence and presence of $CaCl_2$: (●) tubular polymers in the presence of 17 mM $CaCl_2$; (△) tubular polymers in the presence of 10 mM EDTA. Buffers were the same as in the legend of Figure 1.

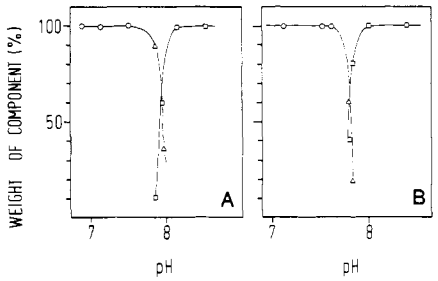

FIGURE 3: Dissociation diagram of tubular polymers at two ionic strengths at 20 °C in the presence of 10 mM EDTA: (A) ionic strength 0.02; (B) ionic strength 0.1; (○) 320S material; (△) 320S–74S material; (□) 3.5S–7.5S material.

Results

Oxygen-Binding Behavior of Tubular Polymers. Figure 1 shows the Hill plots of the oxygen equilibria in the pH range 6.55–7.80 in the presence of 17 mM $CaCl_2$. The binding of oxygen is highly cooperative under these conditions. The maximum Hill coefficient is ~8. The exact value of the Hill coefficient is, however, hard to determine due to the steepness of the oxygen-binding curve. The low-affinity asymptote is highly pH dependent while the high-affinity asymptote is not. In Figure 2, the oxygen affinity is plotted as a function of pH. The polymers display a negative Bohr effect between pH 6.9 and 7.8 in the presence of 17 mM $CaCl_2$. In the absence of $CaCl_2$, oxygen affinity is influenced differently at the pH values studied. Below pH 7, no influence of Ca^{2+} ions was observed. Above pH 7, however, an affinity decrease was measured. No values above pH 7.7 in the absence of $CaCl_2$ could be measured due to the dissociation of the polymers (Figure 3). In the absence of calcium ions the Hill coefficient reached a maximum value of only 2.4 at pH 7.36. Evidently, Ca^{2+} ions increase the cooperativity significantly. The parameters from the fittings are presented in Table I. The fitted curves were in all cases satisfactory since no differences between the experimental points and the fitted curve could be detected by eye (Figure 1).

Table I: Oxygen-Binding Parameters Obtained from Fitting Calculation

pH	r^a	$L_0{}^b$	$P_{50}{}^c$	$h_{50}{}^d$	ΔG^e (kJ/site)
		No $CaCl_2$			
7.19	29.5	9×10^{-5}	1.4	1.5	2.45
7.25	27.8	4×10^{-7}	2.1	2.9	2.63
7.36	34.8	1.6×10^{-6}	1.9	2.4	2.20
7.45	33.3	1×10^{-5}	2.8	2.0	1.92
7.69	42.8	6×10^{-6}	5.0	1.8	1.39
		17 mM $CaCl_2$			
6.88	17.9	2.7×10^{-3}	0.9	1.1	4.73
7.05	14.8	6.2×10^{-7}	1.5	4.9	6.96
7.20	12.8	4.9×10^{-8}	2.8	5.8	7.70
7.39	12.5	6.9×10^{-12}	6.1	7.0	9.03
7.48	14.0	1.7×10^{-14}	11.7	7.6	9.28
7.80	8.4	6.8×10^{-10}	17.7	3.2	8.40

[a] Number of interacting sites. [b] Allosteric equilibrium constant. [c] Oxygen pressure (in mmHg) at which 50% of the sites are occupied with oxygen. [d] Hill coefficient at 50% saturation. [e] Free energy of interaction.

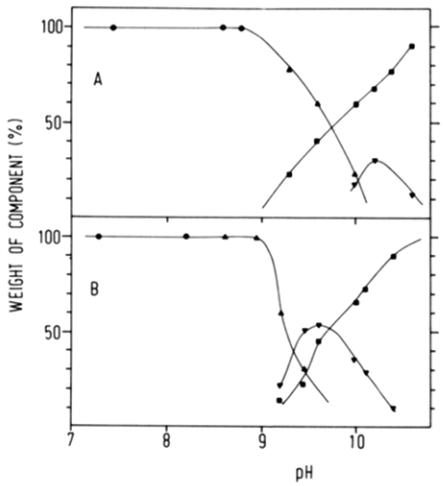

FIGURE 4: Dissociation diagram of tubular polymers at two ionic strengths at 20 °C in the presence of 17 mM $CaCl_2$: (●) 320S material; (▲) 320S–74S material; (▼) 45S material; (■) 3.5S–7.5S material. (A) Ionic strength 0.07; (B) ionic strength 0.15.

Dissociation Behavior of Tubular Polymers. During trypsinolysis of native molecules of β_c-hemocyanin, the collar (Mellema & Klug, 1972) is removed, leading to association of the collarless molecules into tubes of different lengths (Van Breemen et al., 1975). Therefore, one would expect no discrete boundary in the analytical ultracentrifuge. The tubes showed, however, a boundary that had a sedimentation coefficient around 320 S. This value decreased gradually to 74 S with increasing pH. Electron micrographs revealed dissociation into shorter tubular polymers. The sedimentation coefficient of 74 S probably is that of collarless whole molecules. This material dissociates into material having a sedimentation value of 45 S when the pH is increased. At high pH values, the dissociation continued into material with sedimentation coefficients between 3.5–7.5 S. When we had observed this, we analyzed the dissociation behavior further. The results are depicted in Figures 3 and 4. In the absence of Ca^{2+} ions, the tubular polymers dissociate in a very narrow pH range. No material sedimenting with a sedimentation coefficient of 45 S was observed. The influence of ionic strength on the dissociation behavior is of minor importance under these conditions (Figure 3). Addition of Ca^{2+} ions shifts the dissociation of the tubes to higher pH values and broadens the pH range in which it occurs (Figure 4). At low ionic strength, material

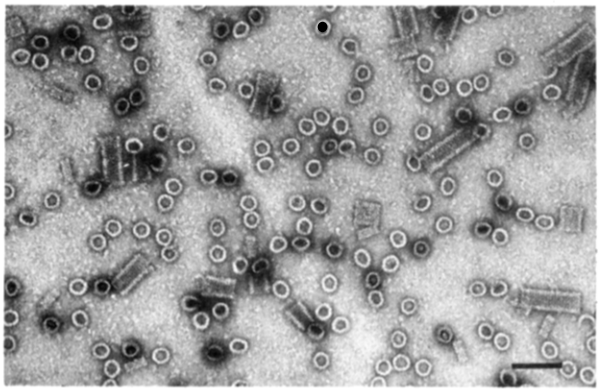

FIGURE 5: Electron micrograph of tubular polymers at pH 9.5 and ionic strength 0.15 in the presence of 17 mM $CaCl_2$. The bar is 100 nm.

with a sedimentation coefficient of 45 S was observed above pH 10. Electron micrographs revealed that this material consisted of half molecules without a collar (Figure 5). At higher ionic strength, the dissociation shifts to lower pH values, and the amount of 45S material increases considerably (Figure 4). At low ionic strength, the polymers dissociate first into material with sedimentation coefficients between 3.5 and 7.5 S; the 45S material appears at higher pH values. At the higher ionic strength, however, the 45S material appears at about the same pH values as the smaller particles.

Discussion

Oxygen-binding in the presence of 17 mM $CaCl_2$ is highly cooperative with a Hill coefficient with the same, or a higher, value than was reported for native hemocyanin (Zolla et al., 1978). The number of interacting sites is lower than found for native hemocyanin, but this is probably due to a slightly different fitting program. For α-hemocyanin, we found r = 10.3 compared with 12 found by Colosimo et al. (1977). Therefore, we conclude that there are no significant differences in the number of interacting sites between the tubes and native molecules although tubes possess two binding sites less per polypeptide chain. Upon removal of the collar and polymerization to tubes, the structural arrangement is changed, but this has no effect on the cooperativity of the system. It may be recalled that some of the peptide bonds between the oxygen-binding domains in the wall, too, are cleaved (van der Laan et al., 1981a). This leads us to the conclusion that a region of interacting sites within a functional constellation does not correspond to a tenth or twentieth molecule but that a functional constellation consists of a set of nearest neighbors. This explanation implies that in a tenth or twentieth molecule cooperative oxygen binding occurs if, and only if, the oxygen-binding domains have the same contacts with each other as they have in whole or half molecules. Cooperativity in a tenth molecule was observed for *Levantina hierosolima* hemocyanin under proper conditions (Shaklai et al., 1975). Cooperative features were also observed in one of the collar domains, which associates to dimers (Torensma et al., 1981a). It seems, therefore, that cooperative features occur when two or more domains, not necessary covalently linked, have the proper orientation toward each other. For *H. pomatia* α-hemocyanin and *Colus gracilis* hemocyanin, cooperativity is only observed when the polypeptide chains are associated into at least half molecules (Van Driel, 1973; Pearson & Wood, 1974). Therefore, the dissociation products do not have the same contacts as they have in the native molecule. This was also observed for *Neptunea antiqua* hemocyanin (van der Laan et al., 1981b). The number of interacting sites within a functional

constellation is pH dependent, as was found for the native molecule, too. In the absence of Ca^{2+} ions the number of interacting sites increases.

Ca^{2+} ions lower the number of interacting sites and increase ΔG per site. Moreover, Ca^{2+} ions keep the protein fixed in one single high-affinity state whereas in the absence of Ca^{2+} ions, several high-affinity states are observed. Tubular polymers and native β-hemocyanin have the same high-affinity state in the presence of 17 mM $CaCl_2$, but unlike native hemocyanin, the polymers possess a low-affinity state with lower affinity resulting in an increased ΔG per site.

The dissociation behavior of the tubular polymers is rather strange. Careful observation of the sedimentation patterns revealed two sedimenting boundaries. The faster moving boundary, with a sedimentation coefficient of ~700 S, is very broad. The percentage material having this sedimentation behavior is dependent on the total hemocyanin concentration. On the contrary, the concentration of the boundary sedimenting more slowly, with a sedimentation coefficient of ~320 S, is almost independent of the total hemocyanin concentration. Increasing the speed of the rotor decreases the sedimentation coefficient of the slowly sedimenting boundary, but the sedimentation coefficient of the quickly sedimenting boundary is increased. The effects of the rapidly sedimenting material are not a reflection of hydrostatic pressure, since the percentage of this material did not change by layering oil on the sample. The sedimentation of the tubes resembles a phenomenon described for DNA that is known as molecular entanglement (Rosenbloom & Schumacher, 1963, 1967; Goldstein & Zimm, 1973). In spite of this complicated sedimentation behavior, it was possible to analyze the dissociation behavior of the tubes. Ca^{2+} ions stabilize the tubular molecules. At the higher ionic strength, ~50% 45S material was observed. Electron micrographs taken from this material revealed molecules ressembling half molecules of native hemocyanin, but since these molecules are dissociation products of tubular polymers, they do not possess a collar. Also, the sedimentation coefficient (45 S) is in agreement with half molecules (sedimentation coefficient 60 S) lacking the collar. Electron micrographs reveal no collar in this material, but sometimes a cap is observed. This indicates that the disputed cap arises from the preparation procedure.

Summarizing, we conclude that the tubular polymers have virtually the same dissociation behavior as native β_c-hemocyanin. However, dissociation occurs at lower pH values, indicating less stability. Since covalent bonds between the oxygen-binding domains are cleaved, this is not surprising. The collar domains in the native molecule probably stabilize tenth molecules since when they are missing, no dissociation products comparable with tenth molecules are observed.

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Registry No. O_2, 7782-44-7; Ca, 7440-70-2.

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Complexation and Phase Transfer of Nucleotides by Gramicidin S†

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ABSTRACT: Gramicidin S (GrS), an amphiphilic cyclosymmetric decapeptide produced by *Bacillus brevis* G-B and Nagano, binds nucleotides in water to yield a complex which partitions into organic solvents. The observed phase-transfer efficiencies at a given pH increase in the order AMP < ADP < ATP. The lipophilic complexes have well-defined stoichiometries, which were determined to be 1:1 for ADP–GrS at pH 7 and ATP–GrS at pH 3 and 1:2 for ATP–GrS at pH 7. The interaction is primarily ionic, involving coordination of the ornithine $N^{\delta}H_3^+$ groups of the peptide and the phosphoryl groups of the nucleotide, with little contribution from the nucleoside moiety. Exchange of organic and inorganic phosphates was also found to be mediated by GrS. The nucleotide complexes are sparingly soluble in water and self-associate extensively in $CHCl_3$, most likely by cross-β-aggregation, to yield large, ribbonlike aggregates which give rise to broad NMR resonances. Structures for the 1:1 and 1:2 complexes are proposed. In the latter, two GrS molecules envelop the nucleotide, orienting their apolar faces externally in opposite directions, while the lateral faces retain considerable polar character and direct aggregation in organic media. The 1:1 complex possesses a single apolar face and is less lipophilic. Binding constants were estimated by simulation of the extraction data. For the 1:1 complexes, $K_{1:1} \simeq 4 \times 10^4$ M^{-1} for either ADP or ATP. Phase transfer of the ATP complex at pH 7 could be modeled either by stochastically independent binding to two noninteracting sites on the nucleotide with $K_1 \sim K_2 \sim K_{1:1}$ or by a sequential process with $K_1 \sim K_{1:1}$ and $K_2/K_1 < 100$. It is concluded that the apparent selectivity of GrS for ATP over ADP is a consequence of the greater lipophilicity and tendency to aggregate of the 1:2 complex, rather than an intrinsically higher binding affinity for triphosphates. GrS is, to our knowledge, the first peptide known to possess phase-transfer activity toward nucleotides; this is, in addition, the first molecular recognition process in which GrS is demonstrated to participate in vitro at physiologically active concentrations.

Considerable attention has been given macromolecules which form stable coordination complexes with ionic substrates in solution. Through judicious placement of charged and polar groups, it has been possible to design complexing agents, or complexones, which bind substrates strongly and with considerable selectivity (Lehn, 1978). Complexones serve as models for the study of biomolecular recognition and transport processes (Lehn, 1978; Tabushi et al., 1978, 1980, 1981) and have important applications in chemical catalysis (Montanari et al., 1982). Much of the recent research on anion complexones of potential biological significance (Dietrich et al., 1979, 1981; Graf & Lehn, 1976; Kimura et al., 1981, 1982) has centered on natural and synthetic polyamines which interact with nucleotides (Dietrich et al., 1981; Kimura et al., 1982; Nakai & Glinsmann, 1977; Tabushi et al., 1980, 1981). Of the latter class of compounds, the simplest examples are the linear amines putrescine, spermidine, and spermine, which form nucleotide complexes that may serve a regulatory function in cell growth (Nakai & Glinsmann, 1977). Macromonocyclic salts containing repeating units of ethylene-, propylene-, and butylenediamine bind nucleotides more tightly than the acyclic ligands (Dietrich et al., 1981; Kimura et al., 1982), presumably because the cyclic compounds are less conformationally mobile and possess a higher charge density. A highly constrained, lipophilic ethylenediamine congener, *N,N'*-distearyl-1,4-diazabicyclo[2.2.2]octane (DS-Dabco),[1] has been shown to bind nucleotides and transfer them efficiently to $CHCl_3$ (Tabushi et al., 1980, 1981). All of these complexes involve ion pairing between the phosphate moieties of the nucleotide and the positively charged amino groups of the ligand and are stabilized by electrostatic and, where possible, hydrogen-bonding interactions.

Gramicidin S [GrS, *cyclo*-(Val[1]-Orn[2]-Leu[3]-D-Phe[4]-Pro[5])$_2$; Figure 1] is a cyclosymmetric decapeptide antibiotic produced by mature cultures of *Bacillus brevis* G-B and Nagano which has been the subject of many physicochemical studies [see Chapter 5 of Izumiya et al. (1979)] but whose biological

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[1] Abbreviations: GrS, gramicidin S; Me_6GrS, [2,2',N^{δ}-trimethylornithyl]gramicidin S; ChaGrS, 4,4'-bis(D-cyclohexylalanyl)gramicidin S; DS-Dabco, *N,N'*-distearyl-1,4-diazabicyclo[2.2.2]octane; AMP, adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; CTP, cytidine 5'-triphosphate; UTP, uridine 5'-triphosphate; TTP, thymidine 5'-triphosphate; 2'-dTTP, 2'-thymidine 5'-triphosphate; 2',3'-dideoxy-TTP, 2',3'-dideoxythymidine 5'-triphosphate; $CHCl_3$, chloroform; $CDCl_3$, deuteriochloroform; CCl_4, carbon tetrachloride; EtOH, ethanol; Me_2CO, acetone; MeOH, methanol; Me_2SO-d_6, dimethyl-d_6 sulfoxide; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; Orn, ornithine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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