Interactions of Poly(N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine) and Poly(N^{δ},N^{δ},N^{δ}-trimethylornithine) with Polynucleotides: Salt Dissociation and Thermal Denaturation†

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ABSTRACT: The interaction of poly(N^{ϵ},N^{ϵ},N^{ϵ}-trimethyl-L-lysine) ($[Lys(Me_3)]_n$) and poly(N^{δ},N^{δ},N^{δ}-trimethyl-L-ornithine) ($[Orn(Me_3)]_n$) with polynucleotides was studied by thermal denaturation, viscosity, and dissociation by salt. The methylated polymers decrease the viscosity of DNA in proportion to the amount of bound peptide. $[Lys(Me_3)]_n$ and $[Orn(Me_3)]_n$ raise T_m of polynucleotides more than do $(Lys)_n$ and $(Orn)_n$. Dissociation of the polypeptide–polynucleotide complexes with NaCl, KCl, or $MgCl_2$ required about half the salt concentration for the methylated polymers as for the parent polymers. The effects of T_m on DNA appear to be complex and may involve differences in the hydrophobic effects, solvation, and conformational entropy. The salt dissociation data are discussed in relation to the role of histone methylation in chromatin function.

Methylated lysyl residues are found in a variety of proteins (Paik & Kim, 1971), including ribosomal proteins and histones. Delange et al. (1969, 1973) have identified N^{ϵ}-monomethyllysine and N^{ϵ},N^{ϵ}-dimethyllysine at residue 20 in calf thymus histone H4 and N^{ϵ}-monomethyllysine, N^{ϵ},N^{ϵ}-dimethyllysine, and N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine at residues 9 and 27 of calf thymus histone H3. Although the function(s) of methylated histone is (are) unknown, Tidwell et al. (1968) and Thomas et al. (1975) have suggested that histone methylation plays a role in maintaining chromatin structure. Baxter & Byyoet (1975) have shown progressive shifts of the ^1H NMR signal from the ϵ and N-methyl carbon atoms, which were attributed to decreased electron density on the CH_2N and CH_3N, in accord with the theoretical calculations of Hehre & Pople (1970). This, they suggested, would increase the affinity of lysyl residues for the negatively charged DNA.

Synthetic polypeptides have been widely used as models to study protein–nucleic acid interactions. $(Lys)_n$[1] in particular has been much used as a model for histones. In order to learn more about the effects of methylation on the interaction of proteins with nucleic acids, we have used $(Lys)_n$ and $[Lys(Me_3)]_n$ as models of proteins that contain methylated and unmethylated lysine residues. We have reported on the synthesis and conformational properties of $[Lys(Me_3)]_n$ and $[Orn(Me_3)]_n$ (Granados & Bello, 1979). We now report on the interaction of methylated and unmethylated polypeptides with nucleic acids. We have studied these interactions by salt dissociation, thermal denaturation, and viscosity, since these methods will give us information on the stability and compactness of the complexes. A subsequent report will describe CD spectra of the complexes.

Experimental Section

Materials. $(Lys{\cdot}HBr)_n$ was purchased from Sigma Chemical Co., calf thymus DNA was from Schwarz/Mann, *Clostridium perfringens* DNA was from Worthington Biochemical Co., and $(Orn{\cdot}HBr)_n$, polynucleotides, and *Micrococcus luteus* DNA were from Miles Laboratories. $[Lys(Me_3)]_n$ and $[Orn(Me_3)]_n$ were prepared as described earlier (Granados & Bello, 1979).

Fluorescent Labeling. To 60 mg of polypeptide in 4.0 mL of buffer (0.05 M Na_2HPO_4, pH 9.5) was added 0.05 mL of dansyl chloride (or fluorescein isothiocyanate) solution (10% in acetone). After 3 h at room temperature, the reaction mixture was centrifuged and the supernate was filtered through a 1.0 × 5.0 cm column of Bio-Gel P-2 with physiologically buffered saline and dialyzed against 2.5×10^{-4} M EDTA (pH 8.0). The amount of label incorporated was estimated by using published extinction coefficients for fluoresceinyl and dansyl conjugates (Chen, 1969). Portions of labeled $(Lys)_n$ preparations were methylated to $[Lys(Me_3)]_n^{dans}$ or $[Lys(Me_3)]_n^{fluor}$. Thus, methylated polymer could be compared with its unmethylated parent.

Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer fitted with a Princeton Applied Physics Model 1108 photon counter. For dansyl conjugates excitation was at 325 nm and emission at 550 nm, and for fluoresceinyl peptides the wavelengths were 495 and 525 nm. Viscosity was measured with a 2-mL Ostwald-type viscosimeter with a buffer flow time of 240 s, at 30 °C. The DNA concentration was 0.06 mg/mL. Spectra and T_m values were measured with a Cary 15 spectrophotometer.

Complexes were prepared by adding polypeptide solution dropwise to an equal volume of nucleic acid solution being swirled on a Vortex mixer. Stock solutions of nucleic acid and polypeptide were at the desired ionic strength before mixing. Complexes were formed at room temperature except with $(dAdT)_n$ and $(dA)_n{\cdot}(dT)_n$, which were done at 5 °C. For T_m measurements solutions were heated in Teflon-stoppered square cross section cells. Temperatures up to 118 °C were achieved since Teflon has a higher coefficient of expansion than does silica and gives a tight seal. No evidence of bubbling was seen.

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[1] Abbreviations used: $(Lys)_n$, poly(L-lysine); $(Orn)_n$, poly(L-ornithine); $[Lys(Me_3)]_n$, poly(N^{ϵ},N^{ϵ},N^{ϵ}-trimethyl-L-lysine); $[Orn(Me_3)]_n$, poly(N^{δ},N^{δ},N^{δ}-trimethyl-L-ornithine); $[Lys(Me_2)]_n$, poly(N^{ϵ},N^{ϵ}-dimethyl-L-lysine).

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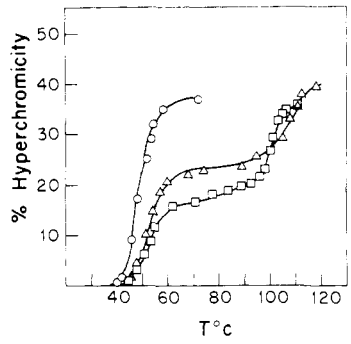

FIGURE 1: Thermal denaturation profiles for calf thymus DNA (○), $(Lys)_n$–DNA (□) (r = 0.45), and $[Lys(Me_3)]_n$–DNA (△) (r = 0.40) in 2.5 × 10^{-4} M EDTA (pH 8.0). Hyperchromicity was measured at 260 nm.

Table I: Melting Parameters of Polypeptide–Nucleic Acid Complexes[a]

polypeptide (M_r)	nucleic acid	r (amino acid/nucleotide)	T_{m1} (°C)	T_{m2} (°C)
	DNA	0.00	48	
$(Lys)_n$ (30 000)	DNA	0.45	52	101
$[Lys(Me_3)]_n$ (30 000)	DNA	0.40	52	108
	$(dAdT)_n$	0.00	20	
$(Lys)_n$ (30 000)	$(dAdT)_n$	0.38	22	81
$[Lys(Me_3)]_n$ (30 000)	$(dAdT)_n$	0.50	22	94
$(Lys)_n$ (30 000)	$(dAdT)_n$	0.40		80[b]
$[Lys(Me_3)]_n$ (30 000)	$(dAdT)_n$	0.44		93[b]
$(Orn)_n$ (28 000)	DNA	0.50	50	102
$[Orn(Me_3)]_n$ (28 000)	DNA	0.50	50	112
$(Lys)_n$ (3400)	DNA	0.50	49	101
$[Lys(Me_3)]_n$ (3400)	DNA	0.48	49	108
$[Lys(Me_3)]_n$ (30 000)	DNA[c]	0.50	91	110
$(Lys)_n$ (30 000)	DNA[c]	0.50	90	103

[a] Solvent was 2.5 × 10^{-4} M EDTA, pH 8, except for experiments of footnote *b*. [b] Solvent was 2.5 × 10^{-4} M EDTA and 10^{-2} M sodium acetate, pH 5.5. [c] These experiments were performed in 0.1 M NaCl, 1.0 × 10^{-3} M Na_2HPO_4, and 1.25 × 10^{-4} M EDTA (*pH 8.0*).

Results

T_m Measurements. Complexes show two transitions: T_{m1}, for uncomplexed DNA, and T_{m2}, for peptide-bound DNA (Figure 1). T_{m2} for $[Lys(Me_3)]_n$–DNA is 7 °C higher than T_{m2} for $(Lys)_n$–DNA (Table I). Similar ΔT_{m2} results were obtained at low salt and in 0.1 M salt. For both, one nucleotide is bound per amino acid residue, since the fraction of the hyperchromicity accompanying T_{m2} is equal to the peptide/nucleotide ratio, r. Table I shows that in all cases methylation raises T_{m2}. The T_m result for $(Lys)_n$–$(dAdT)_n$ is in accord with that of Li et al. (1975). T_{m2} for $(Orn)_n$–DNA is slightly higher than T_{m2} for $(Lys)_n$–DNA, in accord with the result of Olins et al. (1967). $[Orn(Me_3)]_n$ raises T_{m2} 3 °C more than does $[Lys(Me_3)]_n$. The T_{m2} values are the same for polypeptides of 30 000 and 3400 daltons.

In contrast to the increases in T_m with methylated polypeptides, we found the reverse trend for DNA in 0.013 M solutions of chlorides of NH_4^+, $CH_3NH_3^+$, $(CH_3)_2NH_2^+$, and $(CH_3)_4N^+$, for which the T_m values were, respectively, 76, 75, 71, and 68 °C. A similar trend was seen by Gabbay (1966) for T_m values of $(rI)_n$·$(rC)_n$ and $(rA)_n$·$(rU)_n$ complexed with diamines with increasing numbers of alkyl substituents. These trends are opposite to those found with polypeptides.

Hirschman et al. (1967) found that the pK of spermine decreases with higher temperature, with an effect on T_m of DNA–spermine. Chou & Scheraga (1971) and Hermans (1966) found the pK of $(Lys)_n$ to decrease with increasing temperature. Extrapolation to the T_{m2} range would reduce the pK to ~8. $[Lys(Me_3)]_n$ is not titratable; its charge would not be affected by temperature. The T_{m2} values of $(Lys)_n$–$(dAdT)_n$ and $[Lys(Me_3)]_n$–$(dAdT)_n$ at pH 5.5 are 80 and 93 °C, respectively, essentially the same as those at pH 8. Thus, the T_m differences do not result from differences in net charge on the polypeptide.

Base Preference. Leng & Felsenfeld (1966) and Li et al. (1974) found that $(Lys)_n$ binds selectively to AT-rich DNA during gradient dialysis. We did a similar experiment with $[Lys(Me_3)]_n$ at r = 0.5, with mixtures of equal amounts of *M. luteus* DNA (70% GC) and *Cl. perfringens* DNA (30% GC), which melt 21 °C apart, and found that all of the *Cl. perfringens* DNA was covered by peptide while all of the *M. luteus* DNA was free. Thus, for gradient-dialyzed complexes $(Lys)_n$ and $[Lys(Me_3)]_n$ bind selectively to AT-rich DNA.

Dissociation by Salt. DNA binding to histones and $(Lys)_n$ is reversed by high ionic strength, indicating electrostatic stabilization of the complex. We studied the electrostatic contribution in the binding of $[Lys(Me_3)]_n$ to nucleic acids. To detect binding, we observed the increase in fluorescence of dansyl groups conjugated with $(Lys)_n$ and $[Lys(Me_3)]_n$, to the extent of ~1/100 residues. The dansyl group was not methylated, based on the following. The spectrum of $[Lys(Me_3)]_n^{dans}$ was similar to that of $(Lys)_n^{dans}$ and to those of dansylated proteins (Chen, 1969). The specific emission intensity of $[Lys(Me_3)]_n^{dans}$ is the same as that of $(Lys)_n^{dans}$. Methylation to the quaternary ammonium derivative would have affected profoundly the electron delocalization and, therefore, the spectrum and fluorescence. Below pH 5 the emissions of $(Lys)_n^{dans}$ and $[Lys(Me_3)]_n^{dans}$ decrease similarly, probably from protonation of the tertiary amine, the pK of such amines being near 4. If the dansyl group were methylated, it would not be pH sensitive.

When dansylated peptide is added to DNA, the emission increases and the wavelength shifts from 535 to 525 nm, suggestive of a more hydrophobic environment (Stryer, 1968). The change in emission intensity on binding is expressed here as the ratio of the fluorescence of the labeled polypeptide in the presence of nucleic acid to that in its absence, but in the same solvent. The fluorescence of the nucleic acid was insignificant.

The initial increase in ionic strength (0 → 0.3 M NaCl) increases the fluorescence ratio for the complexes (Figure 2). It should be noted that at "zero" ionic strength there is present 2.5 × 10^{-4} M EDTA, 10^{-3} M sodium phosphate, and the counterions of the DNA and polypeptide. At sufficient salt the fluorescence ratio decreases to unity, indicating that the polypeptide is not bound to DNA. Most of the work was done with r = 0.25, but a similar result was obtained at r = 0.75. The fluorescence–[NaCl] curves were nearly the same at 5 min or several days after formation of complexes.

A few experiments were done with fluoresceinyl-labeled peptides, which gave curves (not shown) which are the inverse of those of Figure 2. Binding of $(Lys)_n^{fluor}$ to DNA quenches emission, as observed for clupein^{fluor}–DNA (Wehling et al., 1976). $(Lys)_n^{dans}$–DNA and $(Lys)_n^{fluor}$–DNA dissociate at the same ionic strength. The fluoresceinyl group was used because it contains an anionic carboxylate, which should inhibit binding of the fluorophore directly to DNA. Since the fluorescence–salt curve for $(Lys)_n^{dans}$–DNA is the inverse of that for $(Lys)_n^{fluor}$–DNA, the results are not artifacts of light scattering. Fluorescence polarization of $(Lys)_n^{fluor}$–DNA complexes as a function of NaCl concentration gave a curve (not shown)

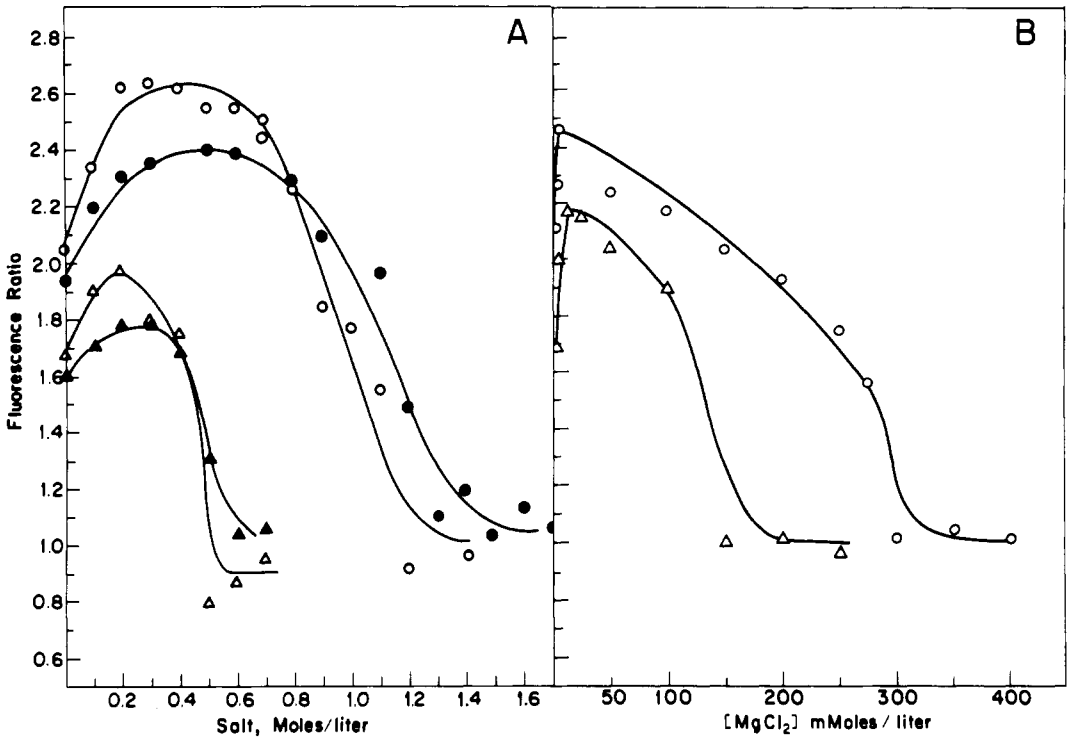

FIGURE 2: Fluorescence ratios of polypeptide–DNA complexes as a function of salt concentration. A: $(Lys)_n^{dans}$–DNA in NaCl (○), $[Lys(Me_3)]_n^{dans}$–DNA in NaCl (△), $(Lys)_n^{dans}$–DNA in KCl (●), and $[Lys(Me_3)]_n^{dans}$–DNA in KCl (▲). B: $(Lys)_n^{dans}$–DNA (○) and $[Lys(Me_3)]_n^{dans}$–DNA (△) in $MgCl_2$.

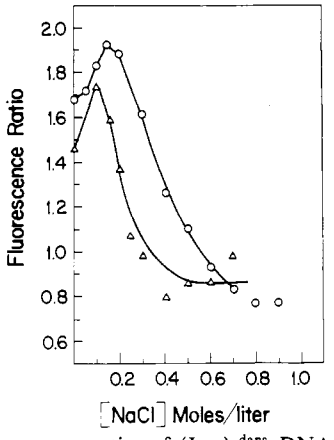

FIGURE 3: Fluorescence ratios of $(Lys)_n^{dans}$–DNA (○) and $[Lys(Me_3)]_n^{dans}$–DNA (△) as a function of NaCl concentration. Polypeptide molecular weight was 3400.

similar to those of Figure 2 for $(Lys)_n^{dans}$–DNA complexes. Evett & Isenberg (1969) reported dissociation curves for $(Lys)_n^{dans}$–DNA (prepared by gradient dialysis) by fluorescence polarization; they found an increase at moderate salt.

With polypeptides of 3400 daltons (Figure 3) dissociation shifted to lower ionic strength. Leng & Felsenfeld (1966) and Akinrimisi et al. (1965) showed that small $(Lys)_n$ dissociates from DNA at lower ionic strength than does large $(Lys)_n$.

For $(Lys)_n^{dans}$–DNA and $[Lys(Me_3)]_n^{dans}$–DNA the ionic strengths at which the fluorescence ratio decreased by 50% and complete dissociation occurred were the same at 25 and 75 °C (not shown). The binding of $(Lys)_n^{dans}$ and $[Lys(Me_3)]_n^{dans}$ to DNA is sensitive to cations (Figure 2) in the order $Mg \gg Na \simeq K$. A feature of the effect of $MgCl_2$ is the sharp rise in the fluorescence ratio at low $MgCl_2$ concentration.

$[Lys(Me_3)]_n^{dans}$ dissociates from denatured DNA at lower ionic strength than does $(Lys)_n^{dans}$ (Figure 4). In contrast

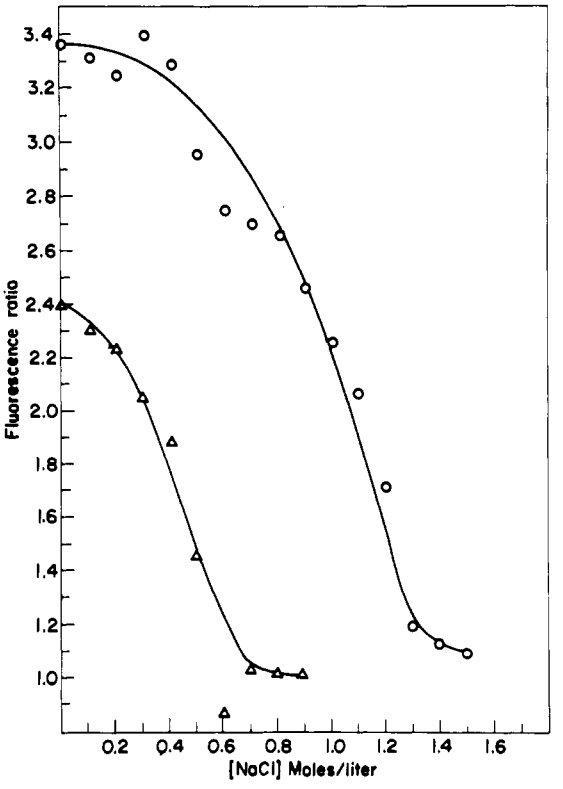

FIGURE 4: Fluorescence ratios for complexes of denatured DNA with $(Lys)_n^{dans}$ (○) and $[Lys(Me_3)]_n^{dans}$ (△) as a function of NaCl concentration. Denaturation was done by heating in boiling water for 5 min and then quenching in an ice bath.

to native DNA complexes, the fluorescence ratio does not increase with the initial increase in ionic strength. Dissociation of $(Lys)_n^{dans}$ from native and denatured DNA occurs near the same ionic strength, in agreement with equilibrium dialysis experiments (Akinrimisi et al., 1965). The decrease in the

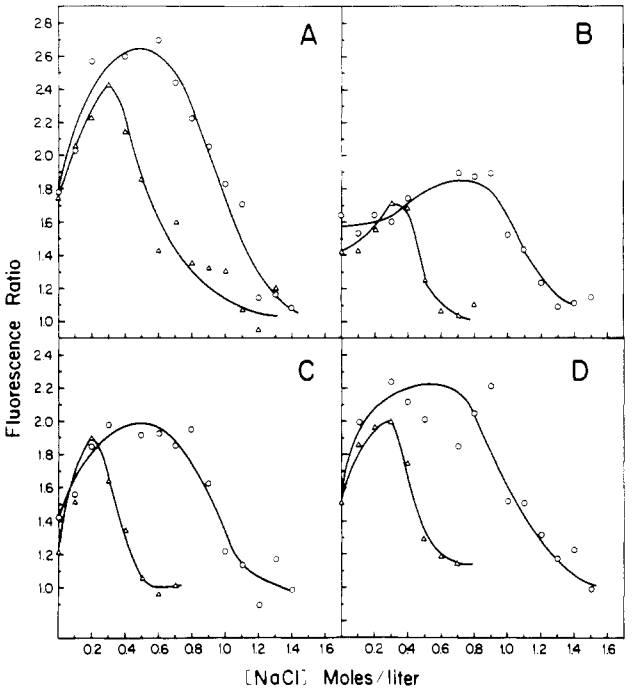

FIGURE 5: Fluorescence ratios for complexes with synthetic polynucleotides. A: $(Lys)_n^{dans}$–$(dAdT)_n$ (○) and $[Lys(Me_3)]_n^{dans}$–$(dAdT)_n$ (△) as a function of NaCl concentration. B: $(Lys)_n^{dans}$–$(dA)_n$·$(dT)_n$ (○) and $[Lys(Me_3)]_n^{dans}$–$(dA)_n$·$(dT)_n$ (△) as a function of NaCl concentration. C: $(Lys)_n^{dans}$–$(dGdC)_n$ (○) and $[Lys(Me_3)]_n^{dans}$–$(dGdC)_n$ (△) as a function of NaCl concentration. D: $(Lys)_n^{dans}$–$(dG)_n$·$(dC)_n$ (○) and $[Lys(Me_3)]_n^{dans}$–$(dG)_n$·$(dC)_n$ (△) as a function of NaCl concentration. $(dGdC)_n$ and $(dG)_n$·$(dC)_n$ were 2.5×10^{-5} M and $r = 0.75$.

fluorescence ratio (not shown) which accompanies dissociation of the complexes of $(DL\text{-}Lys)_n^{dans}$ and $[DL\text{-}Lys(Me_3)]_n^{dans}$ with DNA is more gradual for the DL polymer than for the L polymer, but, here too, half the NaCl concentration is required for $[Lys(Me_3)]_n$ as for $(Lys)_n$. Dissociation of $(DL\text{-}Lys)_n$–DNA occurs over a much wider range of ionic strength than that of $(Lys)_n$–DNA (Shapiro et al., 1969).

The interactions of $(Orn)_n^{dans}$ and $[Orn(Me_3)]_n^{dans}$ with DNA were studied to see the effect of decreasing the length of the side chain. Dissociation of $(Orn)_n$ and $[Orn(Me_3)]_n$ by NaCl is similar to dissociation of $(Lys)_n$ and $[Lys(Me_3)]_n$, respectively (not shown).

In a few experiments we followed dissociation of complexes by light scattering [method of Shiffman et al. (1978)]. The curves were similar to the fluorescence curves. The results for $(Lys)_n$–DNA were in substantial agreement with those of Shiffman et al.

Results with Synthetic Polynucleotides. Salt effects on fluorescence ratios for complexes of $(Lys)_n^{dans}$ and $[Lys(Me_3)]_n^{dans}$ with $(dAdT)_n$, $(dA)_n$·$(dT)_n$, $(dGdC)_n$, $(dG)_n$·$(dC)_n$, and $(rI)_n$·$(rC)_n$ are of the same general character as those for complexes with calf thymus DNA, showing dissociation of $[Lys(Me_3)]_n$ at ~0.4 of the NaCl concentration required for $(Lys)_n$ (Figure 5). There are differences in detail showing that base composition and sequence have some influence. By light scattering Shiffman et al. (1978) found that $(dG)_n$·$(dC)_n$–$(Lys)_n$ required nearly 1.8 M NaCl for complete dissociation, a concentration much larger than that in our fluorescence method, while the complex with $(dAdT)_n$ required substantially less NaCl than that in our experiments.

Nicola et al. (1979) found biphasic dissociation of $(Lys)_n$ from immobilized DNA. By fluorescence, even at our highest r value (0.75), we see no obvious biphasic effect, nor did Shiffman et al. (1978) by light scattering. Nicola et al. suggested that their results arose from more facile dissociation from GC-rich regions. We find that the fluorescence–salt curves for both kinds of dGdC and dAdT polymers are similar with no indication of more facile dissociation of $(Lys)_n$ from the former, but by polarization of fluorescence we find that dissociation of $(Lys)_n$ from $(dGdC)_n$ occurs at ~0.1 M lower [NaCl] than that from $(dAdT)_n$ (not shown).

Table II: Relative Viscosities of Polypeptide–DNA Complexes

complex	amino acid/ nucleotide	NaCl (mol/L)	rel viscosity[a]
$(Lys)_n$	0.25	2.0	1.03
$(Lys)_n$	0.25	1.0	0.82
$(Lys)_n$	0.25	0.1	0.77
$[Lys(Me_3)]_n$	0.25	2.0	1.06
$[Lys(Me_3)]_n$	0.25	1.0	1.04
$[Lys(Me_3)]_n$	0.25	0.1	0.74
$(Lys)_n$	0.50	2.0	1.12
$(Lys)_n$	0.50	1.0	0.53
$(Lys)_n$	0.50	0.1	0.30
$[Lys(Me_3)]_n$	0.50	2.0	1.12
$[Lys(Me_3)]_n$	0.50	1.0	1.18
$[Lys(Me_3)]_n$	0.50	0.1	0.60
$(Lys)_n$	0.75	2.0	1.03
$(Lys)_n$	0.75	1.0	0.31
$(Lys)_n$	0.75	0.1	0.10
$[Lys(Me_3)]_n$	0.75	2.0	1.03
$[Lys(Me_3)]_n$	0.75	1.0	1.2
$[Lys(Me_3)]_n$	0.75	0.1	0.31

[a] Viscosities of the complexes relative to the viscosity of DNA at the same ionic strength. All solutions contained 2.5×10^{-4} M EDTA.

Viscosity. Viscosities of complexes gave verification of dissociation by salt, as shown in Table II. Since histones package DNA compactly, viscosity studies of models are of interest. Table II shows that both $(Lys)_n$ and $[Lys(Me_3)]_n$ decrease the viscosity of DNA in parallel with r. In 0.1 M NaCl at $r = 0.5$ and 0.75, but not at $r = 0.25$, $(Lys)_n$ reduces the viscosity more than does $[Lys(Me_3)]_n$. Presumably, $(Lys)_n$ at high r value produces a more compact structure than does $[Lys(Me_3)]_n$.

Cooperativity. Olins et al. (1968) and Li et al. (1974) found that $(Lys)_n$ bound cooperatively to DNA when the complex was prepared by gradient dialysis from 2 M NaCl. We have found a similar result for DNA–$[Lys(Me_3)]_n$. By cooperative binding we mean that previously bound peptide facilitates the binding of additional peptide to the same DNA molecules.

Discussion

Salt Effects and Viscosity. Baxter & Byvoet (1975), on the basis of the NMR data cited in the beginning of the paper, suggested that methylation would increase the affinity of lysyl residues for DNA. Our results for completely methylated $(Lys)_n$, which cannot form hydrogen bonds, show that electrostatic interactions are stronger for $(Lys)_n$ complexes. But initial work here (unpublished results) on $[Lys(Me_2)]_n$–DNA shows that the NaCl concentration required for dissociation is inversely linear in the number of methyl groups per residue, contrary to the prediction of Baxter & Byvoet (1975).

The changes in fluorescence, polarization of fluorescence, and light scattering with initial increases of salt concentration parallel the results of Bradbury et al. (1975), who showed by NMR that when the basic C-terminal moiety of histone H1 is combined with DNA in zero salt one-fourth of the lysine residues are not bound to DNA, while at 0.35 M NaCl these lysines become bound to the DNA phosphates. They found

similar trends with complete H1 and whole chromatin and that chromatin and H1–DNA contract at the ionic strength at which maximum binding of H1 to DNA occurs. Chang et al. (1973) suggested that moderate increase in ionic strength changes the secondary structure of DNA, which may favor the polypeptide interaction. Wilson & Bloomfield (1979) showed that spermidine and spermine condense DNA to compact particles with enhanced light scattering. The late H. J. Li and co-workers found that most of the scattering from DNA–$(Lys)_n$ complexes at very low salt does not arise from aggregation; but at intermediate NaCl concentrations they found large aggregates as the apparent source of enhanced scattering (J. Howard, personal communication; unpublished results). Moderate ionic strength might enhance compaction and aggregation by reducing repulsion between DNA phosphate groups. The sharp rise in fluorescence at a low concentration of $MgCl_2$ (Figure 5) suggests the importance of neutralizing the DNA charges.

That the salt concentration required to dissociate $(Lys)_n$ and $[Lys(Me_3)]_n$ from DNA is independent of temperature indicates that ΔH for binding to DNA is roughly athermic. The calorimetric results of Giancotti et al. (1975) indicated a ΔH of +300 cal/mol of residues for $(Lys)_n$, where $n = 100$, while the results of Ross & Shapiro (1974) gave a ΔH of −300 cal/mol of residues. Binding of H1 to DNA is athermic or nearly so (Bradbury et al., 1975). Thus, in several respects these simple polypeptides are models for histones. If ΔH is near zero, ΔH of polymer interaction largely cancels ΔH of solvent extrusion, and complex formation is entropically driven by solvent extrusion (Giancotti et al., 1975). The apparent stronger binding of $(Lys)_n$ suggests that more solvent is lost when $(Lys)_n$ binds to DNA. It might be expected that the larger $[Lys(Me_3)]_n$ would cover more DNA and result in more dehydration. We speculate that the bulkier Me_3N group, which cannot approach phosphate as closely as can H_3N, prevents part of the side chain from achieving intimate contact with DNA, resulting in less extrusion of water. Loss of a water molecule from the NH_3^+ of Lys may have a larger $+\Delta S$ than that for $Lys(Me_3)$ since NH_3^+ probably binds water more strongly. Also, loss of the counterions from $(Lys)_n$ and $[Lys(Me_3)]_n$ will have different entropic consequences, since the counterion of the latter should be less strongly bound and, therefore, more normally hydrated.

In contrast to the above results, Nicola et al. (1979) found that dissociation of $(Lys)_n$ and of a copolymer of Lys and Leu from DNA immobilized on Sepharose requires a higher NaCl concentration at higher temperature, indicating an endothermic interaction. Nicola et al. suggested a hydrophobic contribution to the interaction. Since Nicola et al. found a smaller effect between 20 and 37 °C than between 4 and 20 °C and our work was done at 26 and 76 °C, the results might converge if we had used the same temperatures.

The midpoint of the fluorescence–[NaCl] curve of DNA–$(Lys)_n$ is probably not the point of half-dissociation, since it occurs at about the concentration at which the ψ-type CD or ORD spectrum occurs (Ong et al., 1976; Shapiro et al., 1969). [The ψ structure(s) is (are) highly asymmetric polynucleotide aggregates induced by proteins, $(Lys)_n$, other polymers, and salts and which appear to be present in chromatin (Jordan et al., 1972; Sipski & Wagner, 1977; Cowman & Fasman, 1978).] $[Lys(Me_3)]_n$ does not give a ψ-type CD spectrum with calf thymus DNA at any salt concentration (unpublished results), perhaps because the electrostatic stabilization is destroyed before ψ structure can be formed. Therefore, ordinate values along the fluorescent–salt curve for DNA–$[Lys(Me_3)]_n$, especially the descending limb, may not represent structures similar to those of the corresponding ordinate values of the DNA–$(Lys)_n$ curve.

The facile dissociation of $[Lys(Me_3)]_n$ from polynucleotides can be understood readily on the basis of decreased electrostatic interaction because of greater distance between charges and loss of hydrogen bonding between the ammonium and phosphate groups. That $(Lys)_n$ dissociates from all the polynucleotides studied at higher ionic strengths than does $[Lys(Me_3)]_n$ shows that base composition, base sequence, the presence or absence of the 2′-OH group, or the helix type is not a major influence.

Thermal Denaturation. The T_m results for DNA complexes of $[Lys(Me_3)]_n$ and $[Orn(Me_3)]_n$ are in sharp contrast to the results for complexes of $Me_{0-4}N^+$ reported here, complexes with methylated amines reported by Gabbay (1966), and complexes with alkyltrimethylammonium salts reported by Orosz & Wetmur (1977). Only the methylated polypeptides raise T_m over the unmethylated parents. A higher T_m for a complex of DNA with $[Lys(Me_3)]_n$ vs. $(Lys)_n$ or $[Orn(Me_3)]_n$ vs. $(Orn)_n$ can arise from stronger binding of the methylated peptides to native DNA or weaker binding of these to denatured DNA. The lowering of T_m by methylation of small amines is readily explained (if not necessarily fully or correctly explained) by weakening of the interaction with DNA phosphates. This effect is overbalanced by other effects in the cases of $[Lys(Me_3)]_n$ and $[Orn(Me_3)]_n$. T_m is influenced by a number of factors acting on both the native and denatured states: electrostatic, hydrophobic, and van der Waals interactions; hydration, aggregation, and conformational entropy differences. While we can draw few conclusions from the data, we can discuss some of these factors. The salt dissociation data show that electrostatic binding of $[Lys(Me_3)]_n$ to DNA is weaker than that for $(Lys)_n$ below T_{m2}. Perhaps the higher T_m for $[Lys(Me_3)]_n$–DNA arises from relatively weaker electrostatic binding of this peptide to denatured DNA, although it is possible that at high temperature a hydrophobic effect could reverse the situation.

The melting profiles indicate a smaller slope, hence a smaller ΔH, for DNA–$[Lys(Me_3)]_n$. This and the higher T_m mean a smaller ΔS of melting for DNA–$[Lys(Me_3)]_n$. The ΔS of melting has three sources: conformational changes in DNA; conformational changes in the polypeptide; changes in solvation. As to the first of these, Li et al. (1974, 1975) suggested that distortions in the secondary structure of DNA lower its thermal stability. Distortion of DNA by $(Lys)_n$ is indicated by CD changes (Chang et al., 1973), by changes in infrared linear dichroism (Liquier et al., 1975), and by the finding of Lees & von Hippel (1968) that in the complex ~25% of the interbase hydrogens become very rapidly exchangeable. We have found that $(Lys)_n$ distorts the CD spectra of calf thymus DNA and of $(dAdT)_n$ more than $[Lys(Me_3)]_n$ does (unpublished results). As to the second point, when DNA binds to a polypeptide the latter's conformational entropy decreases. $[Lys(Me_3)]_n$ may be more rigid than $(Lys)_n$, as shown by the smaller T_1 relaxation time of the α proton of the former and by the CD spectrum of $[Lys(Me_3)]_n$ being more resistant to elevated temperature (Granados & Bello, 1979). If the difference in conformational entropy between the bound and free states of $[Lys(Me_3)]_n$ is smaller than that for $(Lys)_n$, there will be a relative stabilization of the $[Lys(Me_3)]_n$ complex of native DNA, assuming equal extents of binding of the polypeptides in the denatured state. Involved in this may be the differential rotational entropy of the NH_3^+ and Me_3N^+ groups arising from hydrogen bonding to phosphate in the first but not in

the second. Also, from CPK models we see restricted rotation between the methyl and ϵ-CH_2 and between the methyl and δ-CH_2.

If ΔS of solvation on melting is greater for $(Lys)_n$ than for $[Lys(Me_3)]_n$, it would suggest that DNA–$(Lys)_n$ loses more water or gains less water on melting than does DNA–$[Lys(Me_3)]_n$. Which is correct is not clear since heating can result in a general loss of water–solute interaction but melting exposes more molecular surface for solvation. Also, the data of Herskovits & Bowen (1974) and of Scruggs et al. (1972) show a $+\Delta S$ for transfer of bases from organic media to water, a result which Scruggs et al. suggest may arise from breaking of the water structure. However, the thermodynamic quantities are not known for high temperatures. Thus, it is difficult to assign the ΔS of solvation to the binding of solvent to previously unsolvated base regions or to effects on water. The solvation effects are too complex for drawing conclusions about their roles in determining T_m. The solvation differences affecting T_m include differences in phosphate interactions with NH_2^+ and Me_3N^+, differences in the energetics of counterion release from NH_3^+ and Me_3N^+, differences in the interaction of side chains with DNA arising from steric properties of the NH_3^+ and Me_3N^+ termini, possible differences in peptide group–DNA interactions, and, finally, differences in aggregation.

We have not seriously tried to explain the effect of methylation on T_m by invoking hydrophobicity. Firstly, the trimethylamine groups may not be acting hydrophobically with the less polar parts of DNA but may be restricted to interaction with the phosphate groups. Secondly, we cannot say if the trimethylamine group is more hydrophobically bound to native or to denatured DNA. Thirdly, while the work of Ong & Fasman (1976) and of Mandel & Fasman (1976) with block copolymers of lysine with leucine or valine appears to show that hydrophobic residues stabilize DNA (although not as much as does a lysyl side chain), with the Orn vs. Lys polymers, the smaller, less hydrophobic polymers have the higher T_m values for their DNA complexes. Speculation as to the cause of this would be premature. It would be desirable to study polymers of homologues with shorter and longer side chains.

Relation to Chromatin Function. The facile dissociation of $[Lys(Me_3)]_n$ from DNA by salt suggests that $[Lys(Me_3)]_n$ residues in histones are involved in control of the DNA–histone interaction through ionic changes. Weak interactions would be more susceptible to changes in ionic strength than would be strong interactions. We (Granados & Bello, 1977) have suggested that methylation of histones may weaken DNA–histone interactions. Although dissociation of histones from DNA occurs at NaCl concentrations above physiological, subtler effects occur in the physiological salt range, especially with multiple-charged ions (e.g., Mg^{2+}). Also, with low molecular weight $(Lys)_n$ and $[Lys(Me_3)]_n$ the salt effects are shifted to the physiological range. These polypeptides are of a size comparable to those parts of histones in contact with DNA in chromatin. These studies provide information on completely methylated $(Lys)_n$, a model far from the lightly methylated histones. We are examining models more closely related to histones.

Added in Proof

Methylation of $(Lys)_n^{fluor}$ resulted in methylation of ~75% of the fluoresceinyl hydroxyl groups, based on the absorption spectra at pH 7 (above the pK of fluorescein) and in 0.5 M HCl. The methylated derivative does not absorb at the excitation wavelength used. The quantum yield of $[Lys(Me_3)]_n^{fluor}$ was the same as that of $(Lys)_n^{fluor}$. Therefore, the observed fluorescence originated in $[Lys(Me_3)]_n^{fluor}$ which had not been methylated on the fluoresceinyl groups.

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Solid-Phase Synthesis of Thymosin α_1 Using *tert*-Butyloxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin†

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ABSTRACT: Thymosin α_1 and its desacetyl analogue were synthesized by the solid-phase method. Use of aminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin resulted in an improved yield and allowed the synthetic products to be purified by simple ion-exchange and gel filtration chromatography. Success of the synthesis was largely due to enhanced stability of the peptide-resin linkage to trifluoroacetic acid and to the elimination of hydroxy functions on the resin. This improved quality of the solid support helps eliminate chain loss and chain termination during the synthesis. The purified synthetic peptides were found to be homogeneous by paper electrophoresis, isoelectric focusing in polyacrylamide gel, and thin-layer chromatography. They also had biological activity in the azathioprine-sensitive rosette assay. Use of the new 9-(2-sulfo)fluorenylmethyloxycarbonyl chloride reagent for purification of protected peptides was also demonstrated and discussed.

In the past few years, there have been various reports of the isolation of a family of polypeptides that are believed to play an important role in the function of the thymus gland in the immune system (Friedman, 1975; Bach, 1977). Many of these thymic factors have been suggested to be involved in a hormonal mechanism in which the thymus affects T cell development. A few of these polypeptides have been chemically characterized, sequenced, and synthesized (Bach, 1977). Thymosin α_1, which was isolated and sequenced by Goldstein et al. (1977), was shown to be an acidic peptide whose sequence is as shown in Figure 1. The octaeicosapeptide was shown to be 10–1000 times as active as thymosin fraction 5, from which it was isolated, in vivo and in vitro (Low et al., 1979).

The chemical synthesis of thymosin α_1 has been achieved by both solution- and solid-phase methods (Wang et al., 1979, 1980; Birr & Stollenwerk, 1979). We now report a greatly improved solid-phase synthesis of thymosin α_1. The key to the new synthetic strategy was the use of aminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin as the solid support (Scheme I). The ester linkage between the peptide and this oxymethyl-Pam-resin[1] was previously shown to have enhanced acid stability compared to the usual benzyl ester resin and to prevent loss of peptide chains during the synthesis (Mitchell et al., 1976a). Use of this polymeric support was also shown recently to help avoid trifluoroacetylation (Kent et al., 1979), which has been a major terminating side reaction in solid-phase peptide synthesis. The new synthesis of thymosin α_1 on Pam-resin resulted in an improved yield, and the purified synthetic product has been shown to be homogeneous in a number of chromatographic and electrophoretic systems. The desacetyl analogue of thymosin α_1 was also synthesized and purified (Scheme I), and both peptides were tested for biological activity in the rosette inhibition assay.

Experimental Procedures

Materials

All chemicals were reagent grade. Dichloromethane was distilled from sodium carbonate and DIEA was distilled from sodium hydride. Protected amino acids were purchased from Chemical Dynamics and Vega Biochemicals and were checked for purity by TLC. Unsubstituted resin was copoly(styrene–1% divinylbenzene) beads, 200–400 mesh, from Bio-Rad. Fluorescamine (Fluram) was obtained from Hoffmann-La Roche, [^{3}H]acetic anhydride (50 mCi/mmol) was from New England Nuclear, and PTH-amino acid standards were purchased from Pierce Chemical Co.

Methods

Peptide Synthesis. N^{α}-Boc-amino acids were used in the synthesis. Trifunctional amino acids were protected as N^{α}-Boc-Lys(Tfa), N^{α}-Boc-Thr(Bzl), N^{α}-Boc-Ser(Bzl), N^{α}-Boc-Asp(OBzl), and N^{α}-Boc-Glu(OBzl).

The syntheses of Boc-Asn-4-(oxymethyl)phenylacetic acid and of aminomethyl-resin have been described elsewhere (Tam

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[1] Abbreviations used: Pam, phenylacetamidomethyl; TLC, thin-layer chromatography; PTH, phenylthiohydantoin; Boc, *tert*-butyloxycarbonyl; Tfa, trifluoroacetyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; Sulfmoc, 9-(2-sulfo)fluorenylmethyloxycarbonyl; CF_3COOH, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; high-pressure LC, high-pressure liquid chromatography.

0006-2960/80/0419-3233$01.00/0