A Raman Spectroscopic Study of the Interaction Between Nucleotides and the DNA Binding Protein gp32 of Bacteriophage T4

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Synopsis

Raman spectra of the bacteriophage T4 denaturing protein gp32, its complex with the polynucleotides poly(rA), poly(dA), poly(dT), poly(rU), and poly(rC), and with the oligonucleotides $(dA)_8$ and $(dA)_2$, were recorded and interpreted. According to an analysis of the gp32 spectra with the reference intensity profiles of Alix and co-workers [M. Berjot, L. Marx, and A. J. P. Alix (1985) *J. Ramanspectrosc.*, submitted; A. J. P. Alix, M. Berjot, and J. Marx (1985) in *Spectroscopy of Biological Molecules*, A. J. P. Alix, L. Bernard, and M. Manfait, Eds., pp. 149–154], 1 gp32 contains ≈ 45% helix, ≈ 40% β-sheet, and 15% undefined structure. Aggregation of gp32 at concentrations higher than 40 mg/mL leads to a coordination of the phenolic OH groups of 4–6 tyrosines and of all the sulfhydryl (SH) groups present in the protein with the COO^- groups of protein. The latter coordination persists even at concentrations as low as 1 mg/mL. In polynucleotide–protein complexes the nucleotide shields the 4–6 tyrosine residues from coordination by the COO^- groups even at high protein concentration. The presence of the nucleotide causes no shielding of the SH groups. With Raman difference spectroscopy it is shown that binding of the protein to a single-stranded nucleotide involves both tyrosine and trytophan residues. A change in the secondary structure of the protein upon binding is observed. In the complex, gp32 contains more β-sheet structure than when uncomplexed. A comparison of the spectra of complexed poly(rA) and poly(dA) with the spectra of their solution conformations at 15°C reveals that in both polynucleotides the phosphodiester vibration changes upon complex formation in the same way as upon a transition from a regular to a more disordered conformation. Distortion of the phosphate–sugar–base conformation occurs upon complex formation, so that the spectra of poly(rA) and poly(dA) are more alike in the complex than they are in the free polynucleotides. The decrease in intensity of the Raman bands at 1304 cm^{-1} in poly(rA), at 1230 cm^{-1} in poly(rU), and at 1240 and 1378 cm^{-1} of poly(dT) may be indicative of increased stacking interactions in the complex. No influence of the nucleotide chain length upon the Raman spectrum of gp32[2] in the complex was detected. Both the nucleotide lines and the protein lines in the spectrum of a complex are identical in poly(dA) and $(dA)_8$.

INTRODUCTION

Helix-destabilizing proteins lower the melting temperature of double-stranded polynucleotides. This results from the strong binding to single-stranded polynucleotides combined with a weak binding to double-stranded polynucleotides.[1] Coded by gene 32 of bacteriophage T4, gp32[2] is one of these helix-destabilizing proteins. It is produced in large quantities during phage-DNA replication. It plays a role in the protection of single-stranded DNA from nuclease attack and keeps the single-stranded DNA in a conformation

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suited for the proper functioning of the other proteins active during the replication.

A positive cooperative[3] interaction between gp32 molecules assures complete saturation of the polynucleotide with protein under low salt conditions.[4] An oligonucleotide binding mode can be distinguished from a polynucleotide binding mode by a decreased salt dependence of the effective binding constant for oligonucleotides.[4] This fact, together with an increased mobility of the C-terminal end[5] of the protein upon complex formation, indicates a change of the protein conformation in both the poly- and oligonucleotide binding mode.[4-6]

The gp32 shows a strong tendency to aggregate even at low protein concentrations. It has been suggested[7] that more than one type of protein–protein interaction plays a role leading to aggregates of unlimited size.

Several models have been suggested[4,8-12] for the conformation of the gp32–polynucleotide complex. An electron microscopic study[10] and a hydrodynamic study[9] have established a 50% increase in phosphate–phosphate distance of the polynucleotide in the complex. No agreement exists about the number of nucleotide units covered by one protein molecule. Estimates range from five[11] to eight[13] nucleotides per protein molecule. Recent studies have suggested that gp32 may cover even 10 nucleotides.[14,15] The occurrence of a hyperchromic effect in the polynucleotide absorption spectrum of poly(rA) upon gp32 binding has been interpreted[16] as due to an unstacking of the base residues. The large changes[9,16] in CD spectra can also be explained as a weakening of the coupling of the transition moments[16] of the base molecules. From a combination of hydrodynamic studies and absorption and CD spectroscopy, Scheerhagen[14] has conjectured a detailed model, in which it is suggested that the gp32–poly(rA) complex consists of a helix with a diameter of 1.4 nm, 24 bases per turn, and a pitch of 14.4 nm. This helix is wound into a superhelix with 40 bases per superturn, a diameter of 5.0 nm, and a pitch of 18.4 nm.

The gp32 is a globular protein that consists of one strand of 301 amino acids, the composition and sequence of which are known.[5,6,17] The very large amount of acidic amino acids (about 25–30% is present as aspartic and glutamic acid residues) results in an isoelectric point at pH 5.[6] Among the other residues are 18 phenylalanines, 5 tryptophans, and 8 tyrosines.

The presence of a regular array of tyrosine residues has been demonstrated and may be of importance for nucleotide binding.[17] A nmr study[18] of the nucleotide binding part of the protein complexed with oligonucleotides of varying length has shown that the proton resonances of one phenylalanine, one tryptophan, and five tyrosine residues shift upon nucleotide binding. This provided evidence that some of the aromatic residues are directly involved in complex formation. The importance of aromatic residues for polynucleotide binding also has become clear from chemical studies. After modification of tryptophan[19] or tyrosine,[12] polynucleotide binding was inhibited whereas no changes in gp32 conformation could be discovered.

The effective binding constant of gp32 to polynucleotides is both nucleotide and sugar moiety dependent. It has been reported[13] that, at physiological salt concentrations, poly(dT) binds about seven times stronger than poly(dA) and

that poly(dA) binds about ten times stronger than poly(rA). The reason for differences in binding strength is unknown.

From CD studies it is clear[14] that the bases of the polynucleotide in a poly(rA)–gp32 complex are arranged in a different way than in a poly(dA)–gp32 complex. It is not known whether the protein conformation in both complexes is the same.

It is well known that Raman spectroscopy can give information about the conformation and interaction of polynucleotides and proteins. An advantage of Raman spectroscopy is that, in principle, information about both the protein and the nucleotide part of a complex is obtained. As the nucleotide and the aromatic amino acid residues have clearly recognizable vibrations, it may be expected that information about the role of these residues in complex formation will be obtained. Data about overall conformational changes may also be deduced from the phosphate backbone and amide vibrations.

In this paper we present the results of a Raman spectroscopic study of the complex formation between gp32 and poly(rA), poly(rC), poly(rU), poly(dA), poly(dT), $(dA)_8$, and $(dA)_2$.

MATERIALS AND METHODS

The gp32

The gp32 was obtained as a generous gift from Dr. Blok of the Free University in Amsterdam. It was prepared and purified as in Ref. 6, with a few modifications as described in Ref. 14. The protein was stored at −20°C in a buffer containing 20 m*M* Tris HCL, pH 8, 1 m*M* Na_2EDTA, 1 m*M* 2-mercaptoethanol, 50% glycerol, and 0.1*M* NaCl. The concentration of the protein in the storage buffer was 4–9 mg/mL. Before measurements, the protein was transferred to a buffer containing 10 m*M* sodium cacodylate and 0.1*M* NaCl with pH 7.5, through dialysis during 24 h in Sartorius microcollodion bags. The volume of the protein solution and the protein concentration was then measured using an extinction coefficient of $\epsilon = 36900M^{-1}$ cm^{-1} at 280 nm and a molecular weight of 33487 atomic mass units. Because the desired protein concentration in the measuring buffer was 10 mg/mL, it was always necessary to concentrate the protein after the first dialysis. A second dialysis against the appropriately diluted cacodylate/NaCl buffer assured that, after concentration of the sample under a gentle stream of filtered (Millipore, 0.2 μm) N_2 gas, the composition of the solution was as desired. In the case of measurements of deuterated samples, the same procedure was used. In all experiments a salt concentration of 100 m*M* was chosen such that stoichiometric binding of the poly(oligo)nucleotides takes place.[4] All spectra were recorded at 15°C except for the spectra at high protein concentration, which were measured at ambient temperature ($\approx$ 23°C).

Nucleotides

The nucleotides were obtained from Sigma [poly(rA)] and PL Biochemicals [poly(rU), poly(rC), poly(dA), poly(dT), $(dA)_8$, and $(dA)_2$]. After dissolution, the polynucleotides were dialyzed against the same (measuring) buffer as the protein. The oligonucleotides were used without further purification. The

extinction coefficients used are poly(rA)—$\epsilon = 10000M^{-1}$ cm^{-1} at. 257 nm; poly (dA)—$\epsilon = 9100M^{-1}$ cm^{-1} at 260 nm; poly(dT)—$\epsilon = 8100M^{-1}$ cm^{-1} at 260 nm; $(dA)_8$—$\epsilon = 9980M^{-1}$ cm^{-1} at 260 nm; $(dA)_2$—$\epsilon = 12450M^{-1}$ cm^{-1} at 260 nm; poly(rU)—$\epsilon = 9430M^{-1}$ cm^{-1} at 261 nm; and poly(rC)—$\epsilon = 7150M^{-1}$ cm^{-1} at 269 nm. Spectra of the nucleotides were recorded using the previously mentioned buffer except in the case of poly(rC), which was measured at pH 8.8 to prevent formation of double-stranded poly(rC).[20,21] These spectra were also recorded at 15°C unless otherwise indicated.

The protein/polynucleotide ratio used for the formation of the complexes was one protein to 7 nucleotides.

Raman Spectroscopy

The Raman spectrometer consisted of a Jobin-Yvon HG2S monochromator and a Coherent Argon-ion laser operating at 514.5 nm. The photomultiplier was a Hamamatsu R 943-02. The 632.8-nm line of the He–Ne laser and the 514.5-nm line were used for wavelength calibration.

An LSI-11 computer controlled the measurements. For optimization of the signal intensity, backscatter optics and dual beam passage were used. For samples containing a low protein concentration (10 mg/mL), a high light power of 900 mW was used. No deterioration of the protein occurred as was checked by comparison of spectra from multirun experiments. Slit widths were chosen to give a spectral resolution of 3.2 cm^{-1}. The recorded line maxima had an accuracy of ± 2 cm^{-1}.

Treatment of the Spectral Data

Two band positions at 1004 and 1447 cm^{-1} have been considered as internal intensity references. The CH_2-bending mode at 1447 cm^{-1} has been used in previous studies[22,23] as an intensity reference. Both the 1004- and 1447-cm^{-1} positions have been used to obtain difference spectra in the following way:

$$\{\text{difference}\} = \{\text{complex}\} - \{\text{free polynucleotide} + \text{free protein}\}$$

The complex spectra and the free protein spectrum used to calculate the difference spectrum were all obtained with low protein concentrations (≈ 10 mg/mL). In Fig. 1 a comparison is made between Raman difference spectra using the 1447-cm^{-1} band [Fig. 1(A)] and the 1004-cm^{-1} band [Fig. 1(B)] as a standard. Corresponding positive or negative contributions are indicated with a double arrow. These features will be discussed. Additional positive protein contributions indicated with a single arrow are observed in the difference spectrum for which the 1447-cm^{-1} band was used as an intensity reference. We have concluded that the coefficient used for protein subtraction has been too small in that case and therefore prefer to use the 1004-cm^{-1} band as an internal intensity reference. Another rationale for this choice is that the intensity of this line and its ratio to the intensity of the 1032-cm^{-1} line of phenylalanine is the same in the free amino acid in solution, and in the protein and protein–nucleic acid complexes. The consequence of this choice is

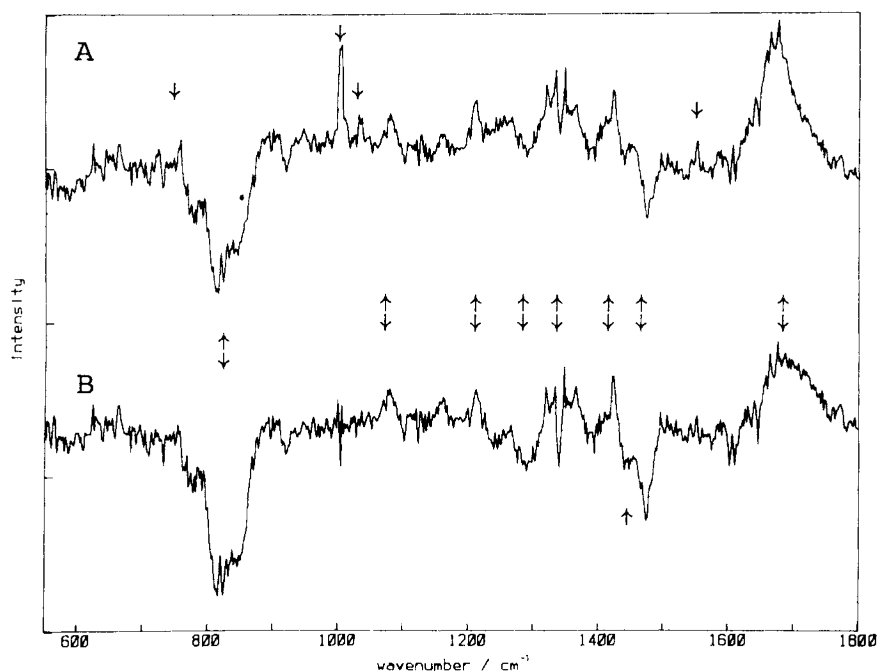

Fig. 1. The effect of the choice of the intensity reference upon difference spectra is illustrated. In A, the band at 1447 cm^{-1} is used. Positive protein contributions remain at 756, 1004, 1032, and 1550 cm^{-1}; these contributions disappear when the 1004-cm^{-1} band is used (B). Other contributions to the difference spectra (indicated with a double arrow) are independent of the choice of either of these bands as an intensity reference and are discussed in the discussion section. It is concluded that in the study of the Raman spectrum of gp32 the band at 1004 cm^{-1} is a more reliable standard than the band at 1447 cm^{-1}.

that a negative contribution arises in the difference spectrum at 1447 cm^{-1} [Fig. 1(B)].

All the changes in the protein and in the nucleotide resulting from complex formation are represented in the difference spectrum. The amount of polynucleotide used in calculation of the difference spectrum was obtained from the chosen protein to polynucleotide ratio. This procedure was checked with samples that contained adenine, using the 1576-cm^{-1} line as an internal intensity reference,[24] and was proved satisfactory.

Interpretation of the Amide I Spectral Region

A curve-fitting procedure using a least squares criterium was used to calculate the contributions of different protein secondary structures to the amide I region between 1630 and 1700 cm^{-1}. The measured spectra were fitted with reference intensity profiles taken from literature.[25,26] Profiles are available for four structures: two types of α-helix conformations called helix A and B, an undefined structure, and the β-sheet structure. After subtraction of the buffer background, described above, a straight line was drawn between 1500 and 1730 cm^{-1}. The part of the spectrum between 1630 and 1700 cm^{-1} was normalized and fitted by the mentioned reference intensity profiles.

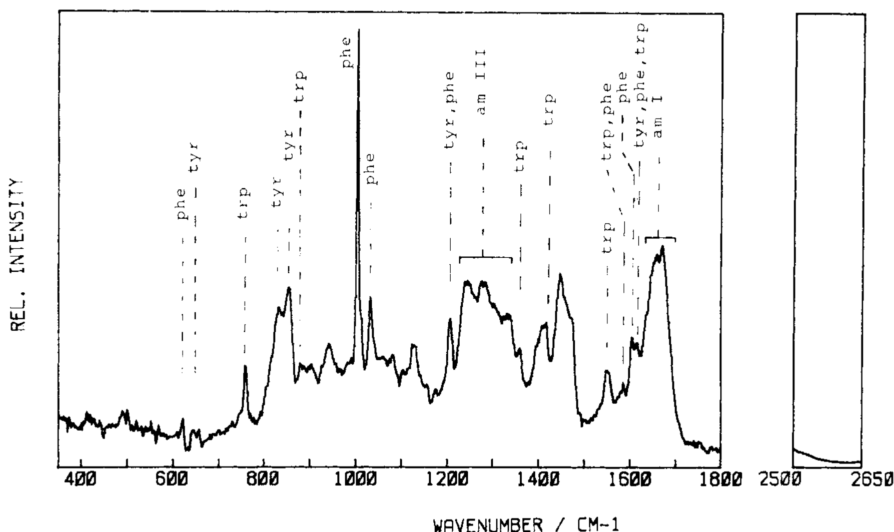

Fig. 2. Raman spectrum of gp32 from 550–1800 cm^{-1} and from 2500–2650 cm^{-1}. The gp32 concentration: 10 mg/mL ($\approx 2.8 \times 10^{-4}$ mol/L); incident laser power: 900 mW; slit widths: 4×400 μm; spectral resolution: 3.2 cm^{-1}; grating interval: 2 cm^{-1}, measuring time per position: 4 s; average of 4 runs, measured in a suprasil cuvette containing 80 μL of solution.

RESULTS

Raman spectra of the free protein and of the protein complexed with nucleotides were measured. In the following sections we present some spectral details. Rather than giving all spectra, we draw attention to only some features.

The gp32

Earlier work describing Raman spectroscopic investigations of proteins[27,28] discovered that the strongest contributions to the spectrum are due to the aromatic residues, i.e., tyrosine, tryptophan, and phenylalanine. Also, vibrations originating in the backbone, and in particular in the amide group, of the protein can be clearly resolved. In Fig. 2 the spectrum of gp32 is presented. Table I gives the assignments according to Refs. 24, 27 and 29–31. We measured gp32 at different concentrations to detect influences of the state of aggregation.[7] Two phenomena are apparent. In the first place no sulfhydryl (SH-) stretch vibration, expected between 2560–2586 cm^{-1}, can be detected in spite of the fact that even at the lowest concentration (1 mg/mL protein) the detection limit should have been exceeded by a factor of 2. At this concentration the threshold for the formation of gp32 multimeres (0.1 mg/mL) is still exceeded, however. It also is well known,[5,6,17] and confirmed by our measurements, that no disulfur bridges, which can be expected between 500–550 cm^{-1} in the Raman spectrum, are present in gp32.

In the second place, a change in the ratio of the intensities of the tyrosine doublet, $I(854/830)$, can be observed. In Fig. 3(A and B) the protein concentration increases from 10 to 40 mg/mL. This change can be explained[32] as a change in the hydrogen-bonding conditions of (a part of) the tyrosine

TABLE I
Position of the Raman bands in the Spectrum of gp32[a]

cm^{-1}	Vibration Due to	Reference
414	α-Helix, Val, skeletal	29
490	Skeletal: Val, Gly	29
622	Phe	29
644	Tyr	29
660	C-S stretch Cys	31
700	C-S stretch Met	24, 29
730	C-S stretch Met	27
744	Ile, Ans. also Thr, Val	30
760	Trp, Val	29, 30
804	Asn	30
812	Ala, Asn	30
834	Tyr; also Gly, Val	29, 30
854	Tyr	29
882	Trp; also C-C stretch and Val	30
904	Ala, C-C stretch	29, 30
928	Thr, Val	29, 30
944	Val, Leu (CH_3 symmetrical rock), Lys, C-C stretch	29, 30
984	Ile	29
1004	Phe	29
1014	Trp	29
1032	Phe, Gly, Ser, Val	29
1058	Lys, Glu, Ser	29
1081	Glu, Thr, C-N stretch	29, 30
1102	Ala, C-N stretch	29, 30
1116	Unknown	
1128	Val, Leu, Ile, Glu, Asp, Gly, C-N stretch	29, 30
1154	C-N stretch	30
1176	Val, Leu, (CH_3 asymmetrical rock)	29
1208	Tyr, Phe	27
1220–1300	Amide III	30
1304	CH_2 twist/wag, C-H def	29, 30
1314	CH_2 twist/wag, C_α-H def	29
1332	CH_2 twist/wag	29
1338	Trp, C-H def	29, 30
1358	Trp, C-H def	29, 31
1396	CH_3 symmetrical def, CH_2 scissor	29
1416	CH_2 scissor	29
1425	Trp	30
1448	CH_2 scissor	29
1460	CH_3 asymmetrical def	29
1468	C-H def, CH_2 symmetrical str, CH_3 deg. str	30
1552	Trp	29
1586	Trp, Phe	30
1604	Phe	29
1614	Tyr, Phe, Trp	30
1630–1700	Amide I	30

[a] The assignments are according to Refs. 24, 27, and 29–31, and references mentioned therein.

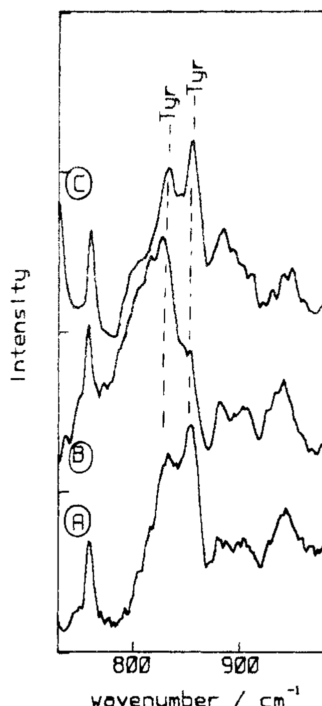

Fig. 3. A comparison of the region of tyrosine doublet (A) low concentration (10 mg/mL), (B) high concentration (40 mg/mL) and (C) high concentration complexed with poly(rA). The "high-concentration" spectra were obtained in a square microcapillary with an internal dimension of 0.5 mm.

residues present in the protein (see the discussion section). An investigation of the temperature dependence of the intensity ratio of the tyrosine lines at 830 and 854 cm^{-1} at low protein concentration revealed no changes, neither in the protein itself nor in complexes with nucleotides.

gp32-Poly(oligo)nucleotide Complex

From the comparison of the spectrum of gp32 complexed with poly(rA) at a high concentration [Fig. 3(C)] with that of gp32 at high concentration [Fig. 3(B)], a change in the intensity ratio $I(854/830)$ again can be observed. The interpretation of this change is presented in the discussion section. As was also observed in gp32 at both high and low concentrations, no SH-stretch vibration could be observed in the high concentration spectrum of gp32 with poly(rA).

We have compared difference spectra (see the materials and methods section) of the complex of gp32 with poly(rA) [Fig. 4(A)], poly(rC), poly(rU), poly(dA) [Fig. 4(B)], poly(dT) [Fig. 4(C)], $(dA)_8$ [Fig. 4(D)], and $(dA)_2$ under conditions of low protein concentration ($\approx$ 10 mg/mL). Several changes in the protein spectrum were observed. These changes did not depend on the kind of nucleotide, the type of sugar (either ribose or deoxyribose), or the length of the nucleotide chain.

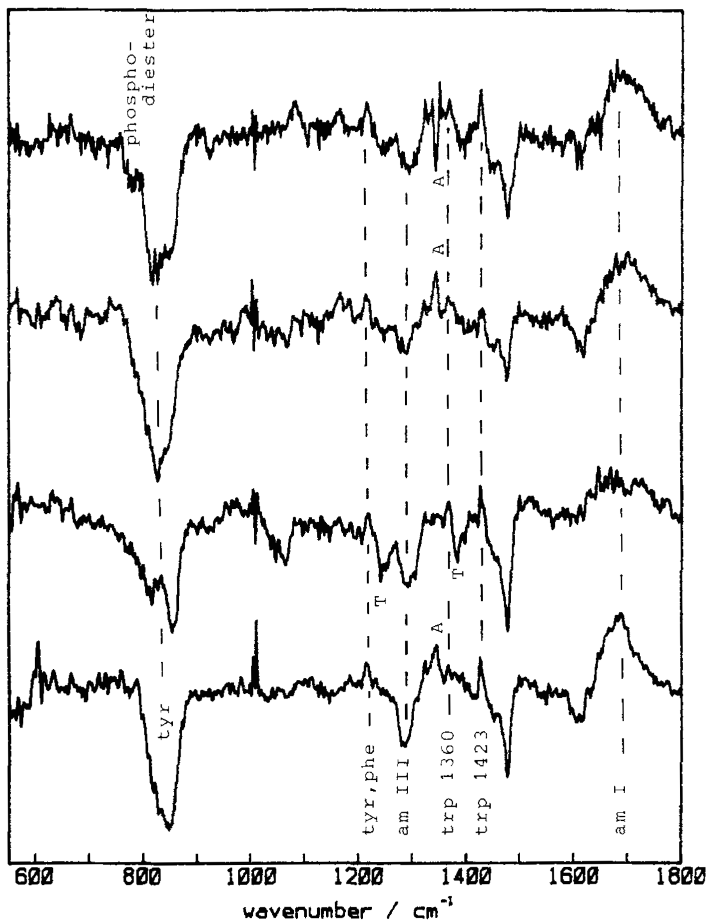

Fig. 4. Difference spectra (see text) for the formation of four poly(oligo)nucleotide–gp32 complexes. (A) gp32–poly(rA), (B) gp32–poly(dA), (C) gp32–poly(dT), and (D) gp32–$(dA)_8$. Spectra of nucleotides and complexes were all obtained under the conditions mentioned in Fig. 1. The following abbreviations are used: T, thymine; A, adenine; Tyr, tyrosine; Trp, tryptophan; Am 1, amide I; and Am 3, amide III.

The following changes can be observed in the protein spectra upon complex formation:

1. At 1366 and 1423 cm^{-1} an increase in the intensity of tryptophan vibrations occurs.

2. A decrease of the intensity takes place at the position of the tyrosine doublet between 810–860 cm^{-1}. The change, resulting from complex formation at low protein concentration, is different from that observed at high protein concentration.

3. An intensity increase occurs at 1208–1212 cm^{-1}. At this position both phenylalanine and tyrosine contribute.

4. A decrease is observed in the intensity of the band between 1445–1470 cm^{-1} due to CH_2-bending and CH_3-bending motions. This decrease is larger at

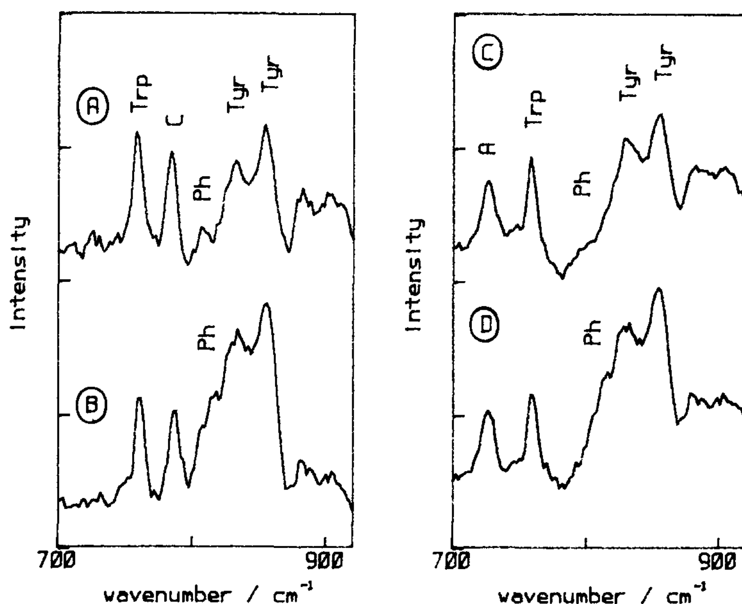

Fig. 5. A comparison of (A) the complex of poly(rC) with gp32 with (B) the sum spectrum of poly(rC) and gp32, and of (C) the complex poly(rA) with gp32 with (D) the sum spectrum of poly(rA) and gp32. Observed in the spectra is the lack of change in the totally symmetric breathing mode of poly(rC) and poly(rA) and the change in the phosphodiester vibration. Abbreviations used as in Fig. 3. C, cytosine; Ph, phosphodiester.

the high-frequency side where CH_3-bending motions are dominant than at the low-frequency side dominated by CH_2-bending motions.

5. Changes in the secondary structure of the protein upon complex formation are indicated by an increase in intensity between 1660–1700 cm^{-1} in the amide I band and a decrease in intensity at 1290 cm^{-1} in the amide III band. Based upon the general characteristics of amide I and amide III bands in proteins, an increase of β-sheet structure has occurred at the expense of other secondary structures present.

A discussion of these features is postponed to the discussion section.

Nucleotides (Base Vibrations)

It should be kept in mind that the changes measured in polynucleotide spectra depend both on the conformation of the polynucleotide in solution and on the conformation of the polynucleotide in the complex. Also, in the complex specific protein–nucleotide interactions may contribute to the observed spectra.

The totally symmetric ring breathing modes of adenine [in poly(rA) at 728 cm^{-1}], cytosine [in poly(rC) at 784 cm^{-1}], uracil [in poly(rU) at 792 cm^{-1}], and thymine [in poly(dT) at 750 cm^{-1}] are neither shifted nor changed in intensity in the complex with gp32 [this can be observed from Fig. 5(A–D) for poly(rC) and poly(rA)].

In the single- and double-bond stretching region, however, some changes can be observed:

Poly(rA) / poly(dA) / (dA)$_8$

A positive shift to 1308 cm^{-1} is observed for the line at 1304 cm^{-1}. This line can be attributed to[33] the (N_9C_8 stretch + N_3C_2 stretch + C_8H bend − C_2H bend) vibration. The intensity of the ($-N_7C_5$ stretch + C_8N_7 stretch) vibration at 1338 cm^{-1} in poly(rA), and 1346 cm^{-1} in poly(dA) and (dA)$_8$, is influenced by protein binding. An increase in intensity takes place in the case of poly(dA) and (dA)$_8$, while a decrease in intensity occurs for poly(rA) [respectively, Fig. 4(B, D, and A)]. This suggests (see the discussion section) that the phosphate–sugar–base structures of poly(rA) and poly(dA) upon binding by gp32 become more alike than in the respective polynucleotides in solution but not completely the same (Fig. 6) and that binding causes a change in the phosphate–sugar–base conformers of both polynucleotides.

The interference of Raman lines of the amide III vibration with the 1304-cm^{-1} line of poly(rA) can be eliminated by deuteration of the samples. The amide III band then shifts[34] from 1200–1300 cm^{-1} to 930–1030 cm^{-1}. The results (Fig. 7) firmly establish the shift of the 1304-cm^{-1} vibration and the decrease in the 1304- and 1338-cm^{-1} vibration. The line of 1576 cm^{-1} has again been taken as an internal reference.[24]

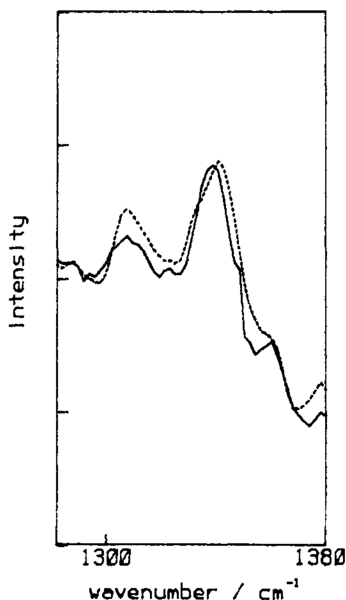

Fig. 6. A detail of the spectra of gp32–poly(rA) (I) and gp32–poly(dA) (II) showing the marker band for stacking interaction near 1304 cm^{-1} and the marker band for the phosphate–sugar–base structure near 1340 cm^{-1}. The data reveal that the conformation of poly(rA) and poly(dA) in the complex are not completely the same. However, they are much more alike than in the free polynucleotides.

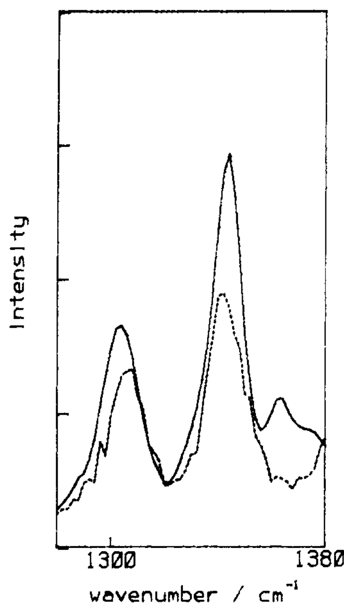

Fig. 7. A comparison of poly(rA) free in solution (solid line) and complexed by gp32 (dotted line) in deuterated samples. The complexed poly(rA) spectrum has been obtained by subtraction of the deuterated gp32 spectrum from the complex spectrum.

Poly(rU)

Free poly(rU) does not have a stacked configuration under our measuring conditions.[35] We measured a decrease in the intensity of the vibration at 1230 cm^{-1} upon complex formation. This is illustrated in Fig. 8, where a comparison is made of the complex spectrum with the sum spectrum.

This vibration is assigned to the (C_5C_6 stretch + N_1H bending + C_5H bending) mode.[36] It is expected that the intensity of this line decreases when stacking interactions occur.[35]

Poly(dT)

Like poly(rU), poly(dT) also lacks significant base stacking in solution.[1] Binding of gp32 leads to a decreased intensity at 1378 cm^{-1} [Fig. 4(C)]. This vibration is assigned to the symmetrical C_5-CH_3 bending motion.[37] It is not clear whether the intensity of this line is sensitive for stacking interactions. Also, the intensity of the (ring-stretch + CH-bending) mode[37] at 1240 cm^{-1} has decreased [Fig. 4(C)]. These changes can also be observed from Fig. 9, in which peak positions of thymine are indicated.

Nucleotides (Phosphate Vibrations)

Both poly(rC) and poly(rA) have a pronounced phosphodiester vibration respectively at 808 and 812 cm^{-1}. In the complex a decreased contribution at these wavenumbers occurs [Fig. 5(A and B) for poly(rC) and Fig. 5(C and D) for poly(rA)]. In the case of poly(rA) a very weak and probably broadened line can be observed at 796 cm^{-1} [Fig. 5(C)] while the stretch vibration of the

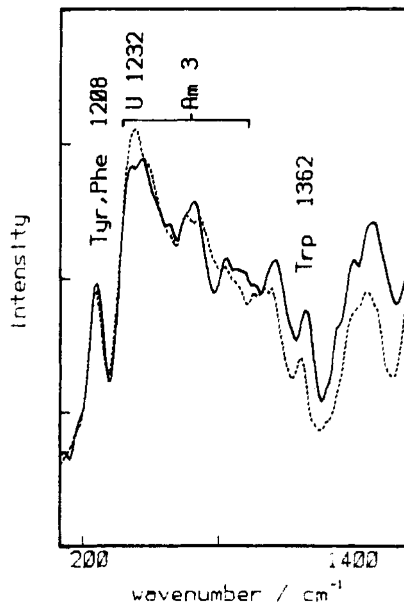

Fig. 8. The solid line is the complex spectrum of poly(rU) with gp32. The dotted line is the sum spectrum of poly(rU) and gp32. From the comparison it can be observed that, as a result of complex formation, a significant decrease in the intensity of the 1230-cm^{-1} vibration of uracil takes place.

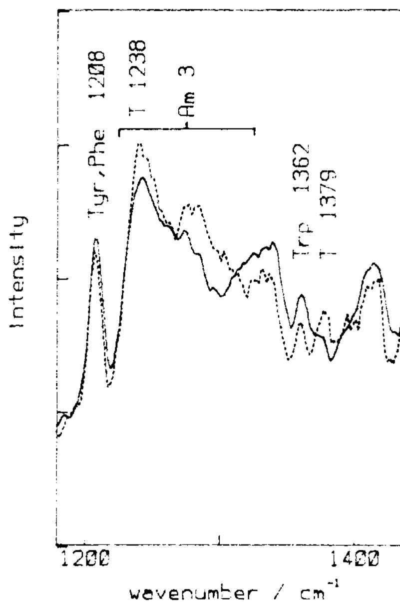

Fig. 9. The solid line is the complex spectrum of poly(dT) with gp32. The dotted line is the sum spectrum of poly(dT) and gp32. The complex formation results in a decrease of the intensity of thymine vibrations at 1240 and 1378 cm^{-1}.

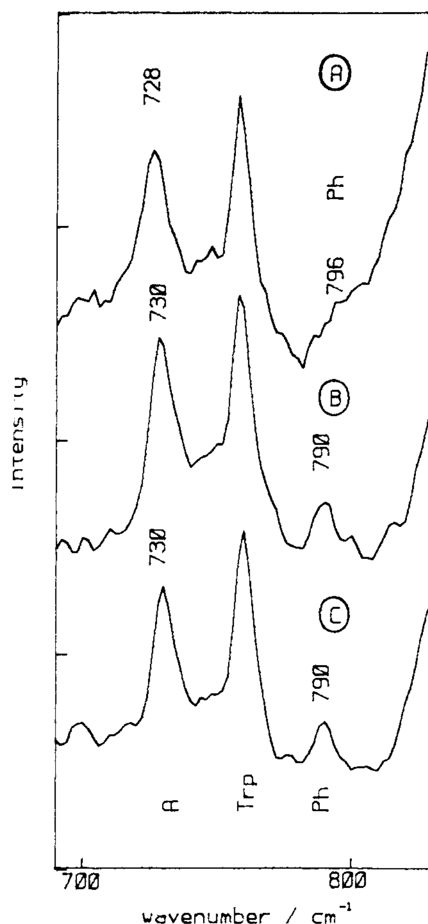

Fig. 10. A detail of the spectra of gp32–poly(rA) (A), gp32–poly(dA) (B), and gp32–$(dA)_8$ (C). The line due to the phosphodiester stretch at 790 cm^{-1} in the case of poly(dA) and $(dA)_8$ is shown, and contrasted with the broad band at 796 cm^{-1} in case of poly(rA). Abbreviations as in Fig. 4.

$(O\text{-}P\text{-}O)^-$ group remains in the same place (not shown). The behavior of the phosphodiester vibration resembles that occurring upon a thermally induced order–disorder transition.[38] In the case of poly(dA) and $(dA)_8$ a band occurs at 790 cm^{-1} in both complex spectra (Fig. 10). This is identical to the position in uncomplexed poly(dA) and $(dA)_8$. The position of this vibration in these compounds is also independent of the temperature over the range of 5–85°C.

DISCUSSION AND CONCLUSIONS

Amide Vibrations

The Raman spectra provide evidence for changes in the secondary structure of the protein upon complex formation, as can be seen from the increase in intensity at 1660–1700 cm^{-1} in the amide I band and the decrease in the amide III region at 1290 cm^{-1} [see Fig. 4(A–D)]. The changes point to an

TABLE II
Results of the Fit of the Amide 1 Region from 1630–1700 cm^{-1} to Reference Spectra[a,b]

	gp32	gp32–Poly(rA)	gp32–Poly(dT)	gp32–$(dA)_8$
α-Helix A	26	24.3	23.8	21.0
α-Helix B	19.3	13.0	16.0	16.6
α-Helix (total)	45.3	37.3	39.8	37.6
β-Sheet	39.5	44.6	46.1	48.1
Undefined	15.2	18.1	14.1	14.3

[a] The numbers give percentages each particular secondary structure present in free gp32 and in gp32 in a complex with poly(rA), poly(dT), and $(dA)_8$.

[b] The reference spectra from Refs. 25 and 26 are used. Two reference spectra for α-helix-type secondary structure are used in order to be able to fit a variety of α-helix band positions and bandshapes. These band positions may range from 1640–1654 cm^{-1}. The peak positions of the used reference spectra are α-helix A: 1640 cm^{-1}; α-helix B: 1652 cm^{-1}; β-sheet: 1673 cm^{-1}; and undefined: 1660 cm^{-1}. The fundamental parameter for the amount of α-helix present is the total amount of α-helix. The reference spectra were obtained Refs. 25 and 26 from a set of proteins with a secondary structure known from x-ray crystallography. The Raman spectrum of proteins from this set could be accurately fitted by the obtained reference spectra. The results of the fit of Raman spectra correlated well with those obtained from crystallography. A correlation coefficient better than 0.96 was obtained. A correlation coefficient of 1 signifies an exact coincidence of Raman and x-ray results.

increased amount of undefined and/or β-sheet at the expense of α-helix secondary structure. From these changes we can conclude that the protein adapts to the presence of the nucleotide. A change of the protein structure has been proposed[5] to account for the increased accessibility of the COOH-terminal domain in the polynucleotide binding mode. The increase of β-sheet structure that we observe (Table II) occurs irrespective of a binding in the polynucleotide or in the oligonucleotide binding mode. This is also neither dependent on the kind of nucleotide nor on the length of the nucleotide chain. So we are led to the conclusion that the binding of any nucleotide induces this change in the protein irrespective of the length of the nucleotide binding site being occupied. Therefore this effect is not a result of protein–protein interactions in the cooperative binding mode. The observation in Ref. 5 that no increased accessibility of the COOH-terminal domain takes place in case of oligonucleotide binding rules out the possibility that it is merely a change in mobility of this domain.

The presence in gp32 of a large amount of aspartic acid, glutamic acid, asparagine, and glutamine allows us to propose that at least part of the changes in the amide I band (predominantly those at the high-frequency side) arises from alterations in the side bands of these amino acids upon nucleotide binding. Because there is no indication that changes in the amino acids may also affect the region around 1290 cm^{-1} in the amide III band it is reasonable to maintain that a change in the secondary structure does occur. With respect to the amide I band we cannot preclude, however, that both effects can be traced in the difference spectrum.

The results of the curve fitting of the amide I band (Table II) are quite different from those previously obtained from UV CD,[8,12] which amounted to 22% α-helix, 26% β-sheet, and 52% unordered (undefined) conformation. The

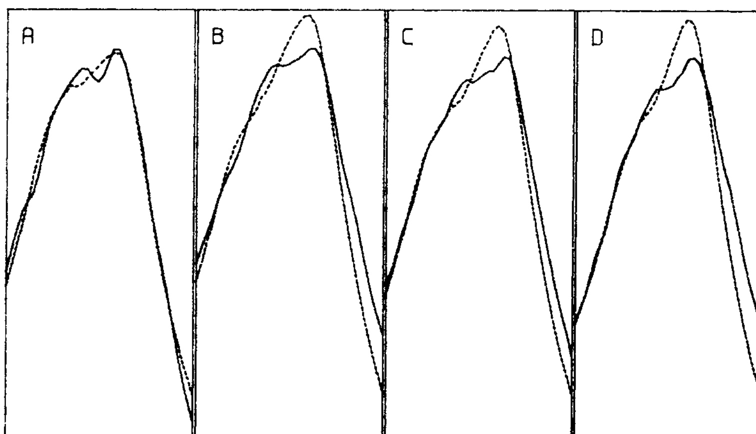

Fig. 11. An analysis of the amide I band from 1630 to 1700 cm^{-1}. (A) gp32, (B) gp32–poly(rA), (C) gp32–poly(dT), and (D) gp32–$(dA)_8$. The solid line represents the measured spectrum. The dotted line is the result of the fit using the reference intensity profiles of four secondary structures determined by Alix and co-workers.[25,26]

results of our curve fitting (Table I) indicate the presence in the protein of 45% α-helix, 40% β-sheet, and 15% undefined structure. From Fig. 11 it can be seen that the fine structure in the gp32 spectrum could not be "ideally" fitted.

The solution to the discrepancy between the results of fitting procedures in Raman spectroscopy and CD must be sought in a refinement of the reference intensity profiles for both types of spectroscopy probably leading to a larger amount of distinct secondary structures, already proposed in literature.[39]

Tryptophan

An investigation of Bence–Jones protein[22] and filamentous viruses[29] has led to a correlation between spectral details related to tryptophan and its surroundings. It has been proposed[22] that a high intensity ratio of the tryptophan vibrations at 879 and 759 is indicative of hydrophobic surroundings of the tryptophan residue. A clearly resolved and consequently sharp tryptophan vibration at 1359 cm^{-1} has also been proposed as an indicator for hydrophobic surroundings of the tryptophan residue.[29,30,40]

In gp32 the tryptophan lines at 759, 879, 1359, and 1552 cm^{-1} are at identical positions as for tryptophan in aqeous solution. Neither the intensity of the 879 cm^{-1} nor the intensity of 1359 cm^{-1} indicates hydrophobic surroundings of tryptophan residues.

In the complexes with poly(oligo)nucleotides also, no change in the position of the tryptophan lines is observed. Therefore, from the line position no change in the surroundings of the residues can be concluded. Stacking interactions, however, do not necessarily lead to shifted frequencies.[40] The difference spectra [Fig. 4(A–D)] indicate an increase in intensity at two positions where tryptophan lines contribute: 1358–1362 and 1420–1425 cm^{-1}. The change in the intensity of the vibration at 1360 cm^{-1} may indicate[40,41] an increase of hydrophobicity of the surroundings. Also, the interaction of nucleophilic groups on the indole N-H bond can lead to a corresponding effect.[22] The

involvement in the gp32–nucleotide interaction of the corresponding pyrrole ring system of the tryptophan molecules can be seen by virtue of the increase in intensity near 1422 cm^{-1}. It is to this line as well as to the line at 879 cm^{-1} that the imidazole N-H-bending motion contributes.[42] In the previously mentioned study[22] no change occurred in the line near 1422 cm^{-1}. In our study, however, protein binding to a nucleotide lattice does not influence the intensity of the 879-cm^{-1} vibration. This difference may be due to the fact that the 879-cm^{-1} vibration is more connected to the benzenelike part of the molecule while the 1422-cm^{-1} line is connected to a stretch motion of the pyrrole ring system. We therefore conclude that an interaction of tryptophan with the nucleotide takes place via the pyrrole ring.

Tyrosine

A region of particular interest in protein spectra is the region between 800 and 870 cm^{-1}, because of the occurrence of two lines due to tyrosine. The relative intensity of these lines depends on the degree of hydrogen bonding to the phenolic hydroxyl group. Hydrogen bonding influences the electron density of the phenolic hydroxyl group, thereby changing the relative position of a symmetric ring breathing mode (ν_{1a}) and a nonplanar ring vibration (ν_{16a}).[32] The relative intensity of the doublet is related because of a Fermi resonance between the fundamental of the ν_{1a} vibration and the first overtone of the ν_{16a} vibration. Therefore it follows that the relative intensity can be used as a measure of the hydrogen-bonding interactions of the phenolic OH group. An empirical relationship has been established.[32] Using the intensity ratios presented in Ref. 32 for different types of hydrogen-bonding interactions, the amount of tyrosine residues for which the surroundings change when the protein concentration increases (Fig. 3) can be calculated. It is thereby assumed that no ionized species are present, (the pH being 7.5) and that no phenolic hydroxyl group acts as an acceptor of strong hydrogen bonds, a situation that occurs, for instance, in solid L-tyrosine · HCl. From such an analysis it follows that, for gp32 at low concentration, all eight tyrosine residues are involved in hydrogen bond donating or accepting interactions of moderate strength, whereas at high concentration a change in 4–6 tyrosine residues resulting from coordination by strong hydrogen-bond acceptors is noticed (see Fig. 3). This number of residues is in reasonable agreement with the observations from nmr spectroscopy[18] and from a chemical modification study,[12] which show that five tyrosine residues are involved in binding of polynucleotides. These results can be explained in the following way: In gp32 carboxyl groups from aspartic and glutamic acid are present in abundance. At least part of these groups will be present at the protein–solution interface (C. Otto, (unpublished results) to take part in favorable ionic interactions with the polar solution. Together with the tendency of the protein to form multimer aggregates[7] it is quite likely that some carboxyl groups from neighboring gp32 molecules modify the surroundings of tyrosine residues at high protein concentrations. This also indicates that the tyrosine residues are present at the outside of the protein molecule. The ability of polynucleotides [Fig. 3(C)] to shield the 4–6 tyrosine residues from the coordination by carboxyl groups makes it likely that the tyrosine residues are involved in the

nucleotide interaction and also that the nucleotide binding site is at the surface of the gp32 molecule.

The difference spectra (from protein and protein–nucleotide complexes measured at a low concentration of 10 mg/mL) also reveal an overall intensity decrease between 800–870 cm^{-1} together with small additional changes in shape and position of the tyrosine doublet. The peak positions of the doublet in the case of gp32 are 834 and 854 cm^{-1}, while for gp32 in complex with poly(rA) the positions are shifted to 830 and 856 cm^{-1}, respectively. These changes in position are most likely the result of the cumulative effect of complex formation on several tyrosine residues (8 residues are present), which cannot be separated in the Raman spectrum. The measured intensity decrease between 800 and 870 cm^{-1} occurs irrespective of the kind of binding mode, the type of nucleotide base, or the chain length, and therefore seems to correspond to a change in the protein as soon as binding occurs. The origin of this change is not, however, understood.

1208 cm^{-1}

The difference spectra reveal a positive contribution at 1208–1210 cm^{-1}. Both phenylalanine and tyrosine residues contribute to the line at this position. Neither can be excluded from being affected by polynucleotide binding. From amino acid spectra the contribution of each phenylalanine residue is estimated to be $2\frac{1}{2}$ to 3% of the measured intensity. Many phenylalanines must be influenced by the binding to allow for a change of about 6–8% in the protein spectrum. Each tyrosine residue contributes about 5–6% so a smaller partial change than in the case of phenylalanine is necessary to account for the observed change.

Assuming that the surroundings of five tyrosine residues have changed in a similar manner, a change of 20–60% in the 1208-cm^{-1} line of each of these five residues would explain the observed change.

Methyl / Methylene-bending Modes

A large number of methyl and methylene groups, respectively 136 and 375, are present in gp32. In the region between 1440 and 1480 cm^{-1}, where these groups contribute, a decrease in intensity occurs when complex formation takes place. Again, the change seems independent of the kind of nucleotide with which binding occurs and seems higher at the high wavenumber side of the band where methylene-bending contributions dominate the methyl-bending mode. The intensity decrease in this region is still quite puzzling. The many amino acids possibly involved hamper a further interpretation.

Sulfhydryl Groups

The gp32 contains four cysteine residues. We have tried to detect the strong SH-stretch vibration ($\approx$ 2575 cm^{-1}) at concentrations as low as 1 mg/mL gp32. No signal was obtained, while the SH concentration should still be a factor of 2 above the detection limit. At this concentration we are still a factor of 10 above the threshold for aggregation, which is roughly at 0.1 mg/mL.[7] The explanation may be similar to that for the changes observed for the

tyrosine residues. Coordination of the sulfhydryl groups by carboxyl or other groups may be responsible for the absence of a SH-stretch vibration.

That the supposed coordination of the SH groups still exists when no such effect can be measured for the tyrosine residues may be explained by the acidic strength of the different groups, i.e., SH: $pK_z = 8.3$; Phenolic–OH: $pK_z = 10.1$.[35]

Also, because no protein concentration dependence of the SH vibration has been observed, the possibility that the SH groups are coordinated by groups of the same protein molecule to which the SH groups belong cannot be neglected.

That no change in SH coordination is found upon polynucleotide binding is the more surprising since three of the four cysteine residues are found between a more or less regular array of six tyrosine residues in the linear amino acid sequence.[17] This array may be involved in nucleotide binding.

Polynucleotides

When comparing the Raman spectra of single-stranded polynucleotides with those complexed by proteins we must realize that several distinct interactions may influence the relative intensity of the base vibrations. In the first place, there is the possibility that hydrogen bonding influences the intensity of base vibrations.[43] Protein subunits may, for instance, form hydrogen bonds with those positions on the base that are involved in hydrogen-bonding interactions in double-stranded nucleotides. In the second place, the protein may induce a certain type of sugar puckering with either *syn* or *anti* conformation of the base, thereby giving rise to a special phosphate–sugar–base conformer. This conformation does not necessarily have to be a conformation known from the investigations of double-stranded structures.[44,45] In the third place, interactions between the electron clouds of (partially) stacked bases also determine the measured relative intensity. The protein may influence these stacking interactions in several ways. We will return to this point later. It is clear that with Raman spectra alone these possible causes of the change of the relative intensities cannot be clearly distinguished from each other.

When the spectra of the uncomplexed single-stranded poly(rA) and single-stranded poly(dA) are observed under the same conditions, the intensity of the band near 1340 cm^{-1} is larger in poly(rA) than in poly(dA). Furthermore, the position of the (N_9C_8 stretch + N_3C_2 stretch + C_8H bend − C_2H bend) vibration is 1304 cm^{-1} in poly(rA) and 1308 cm^{-1} in poly(dA). The peak height of this band is larger in poly(dA) than in poly(rA). These spectral differences are due to the second and third type of interaction. The sugar puckering in the case of poly(rA) is C_3'-*endo* while it is C_2'-*endo* in the case of poly(dA). The orientation of the base is "*anti*" in both cases. [For a definition of the types of puckering and the *anti* (*syn*) conformation, see for instance, Ref. 46.] The degree of base stacking of free polynucleotides can be influenced by changing the temperature of the solution. At 85°C both poly(rA) and poly(dA) are unstacked while their Raman spectra are not the same. This must be due to the conformation of the phosphate–(deoxy)ribose–base conformer. The most notable difference between poly(rA) and poly(dA) is, then,

the intensity of the line near 1340 cm^{-1} attributed to the ($-N_7C_5$ stretch + C_8N_7 stretch) vibration. The intensity of this line decreases in poly(rA) and is independent of the temperature in poly(dA). These observations indicate that this line is a marker for the structure of the phosphate–sugar–base conformation rather than for stacking interactions, because an intensity increase would have been expected if stacking interactions were the dominant interaction to describe the observed temperature-dependent intensity of these vibrations.[35]

The line at ≈ 1340 cm^{-1} can easily be observed in the complex. Binding of gp32 to the polynucleotide leads to a decrease in intensity for this line in the case of poly(rA), while in case of poly(dA) and $(dA)_8$ this line increases in intensity. We might think, from the similar influence that gp32 binding and temperature increases have on the intensity of the vibration near 1340 cm^{-1} in poly-rA, that binding of gp32 influences the structure of poly(rA) in a way similar to a temperature increase. This point of view is, however, not in agreement with what can be observed for gp32 binding and temperature increases in the case of poly(dA) and $(dA)_8$. This behavior can be understood when we suppose that gp32 not merely causes an increase in base–base distance but also changes the phosphate–sugar–base conformation in case of poly(rA). Although the conformation of the nucleotides cannot be deduced from the data, it may be concluded that the structure of the phosphate–sugar–base conformation in poly(rA) and poly(dA) in the complex resemble each other more closely than in solution, but are not completely alike (Fig. 6). It may be that the large decrease in intensity in the 1340-cm^{-1} line of poly(rA) complexed by gp32 (Fig. 7) is due to a change of sugar puckering from C_3'-*endo* to C_2'-*endo*. The increase in the intensity of this line in poly(dA) [and $(dA)_8$], may then be the result of the greatly increased base–base distance in gp32–polynucleotide complexes. The remaining difference (Fig. 6) between the poly(rA) and poly(dA) Raman spectra may be a direct effect of the OH group on the ribose in the case of poly(rA). A comparison of free poly(rA) and poly(dA) at low and high temperature shows that the magnitude of the intensity change is largest for the vibration at 1304 cm^{-1}: the (C_8N_9 stretch + C_2N_3 stretch) vibration.[33,46] Thus this band can be regarded as a marker band for stacking interactions.

We can discern several ways in which a protein can influence stacking interactions of a polynucleotide: (1) by changing the base–base distance, which occurs in a gp32–polynucleotide complex[9,10]; and (2) by the introduction of aromatic amino acids like tyrosine, tryptophan, and phenylalanine between the nucleotide bases.

Following the stacking interactions by observation of the behavior of the 1304-cm^{-1} vibration in poly(rA) (Fig. 7), it can be noted that the intensity of this vibration decreases upon binding by gp32. This behavior suggests that increased stacking interactions can occur in the complex. This is in contrast to what we would expect from the increase in the base–base distance that gp32 induces.

Another possibility, however, is the stacking of aromatic residues of the protein between the nucleotide base planes. When gp32 binds poly(rA), an increase of the base–base distance from 2.8 to 4.6 Å[14] takes place. Insertion of aromatic amino acids between the base planes would decrease the distance of an adenine base to the next aromatic residue from 2.8 to about 2.3 Å. So it is

very well possible that we observe, in the decrease of the vibration at 1304 cm^{-1}, the stacking of aromatic residues between the nucleotide bases leading to increased stacking interactions. This interaction may also be responsible for the decrease in intensity of the vibration at 1230 cm^{-1} in poly(rU) and of the vibrations at 1240 and 1380 cm^{-1} in poly(dT).

In this section we relate some of our conclusions with those obtained from other spectroscopic studies. Nuclear magnetic resonance studies[18] have previously revealed that tyrosine, phenylalanine, and one or more tryptophan residues are influenced by nucleotide binding, probably through stacking interactions. This must be compared with our finding that a stacking marker band in poly(rA) indicates the increase of stacking interactions when poly(rA) forms a complex with gp32.

We have observed, both through changes of the vibration spectrum of the backbone, as well as through an intensity increase of the 1336-cm^{-1} band in poly(rA) and an intensity decrease of the 1340-cm^{-1} band in poly(dA), that a change occurs in the phosphate–sugar–base conformer. Previous studies[9,10] have established an elongation of the nucleotide backbone upon gp32 binding and this must be coupled to changes in the phosphate–sugar–base conformer.

The Raman spectra of uncomplexed poly(rA) and poly(dA) show large differences. In a complex with gp32, however, the spectra in the region of the phosphate–sugar–base conformer reveal a near coincidence of these spectra. This may be compared with the results from a CD study[9] where a better similarity was also observed between poly(rA) and poly(dA) in the complexed state than in the free state. It can therefore be stated that the protein induces comparable secondary structures in these polynucleotides.

SUMMARY OF CONCLUSIONS

The following conclusions regarding the protein and the nucleotides can be drawn:

1. The measurements at high protein concentration reveal in an indirect way the involvement of 4–6 tyrosine residues in nucleotide binding.
2. All SH groups of gp32 are coordinated. No effect of protein concentration on the coordination can be measured above 1 mg/mL.
3. The nucleotide binding region of gp32 is situated on the solution surface of the protein.
4. The pyrrole ring system of one or more tryptophans is influenced by complex formation.
5. An intensity increase is observed for the vibration at 1208 cm^{-1}. It is most likely attributed to a change in the tyrosine residues.
6. The secondary structure of gp32 changes. After complex formation an increased contribution of β-sheet structure is present. The conformation change does not depend on the length of the nucleotide chain.
7. The intensities of methyl- and methylene-bending modes are decreased as a result of complex formation.
8. The change in the phosphodiester-stretch vibration of poly(rA) and poly(rC) resembles the behavior upon order–disorder transition.
9. The phosphate–sugar–base structure in poly(rA) and poly(dA) changes when binding by gp32 occurs.

10. With respect to the phosphate–sugar–base structure, it can be remarked that poly(rA) and poly(dA) resemble each other more closely when bound by gp32 than when free in solution. A sugar dependence of the conformation still exists.

11. Increased stacking interactions due to intercalation of tyrosines can explain the decrease in intensity in a stacking-marker band in poly(rA). Stacking interactions may also account for the decreased intensity of vibrations in poly(rU) and poly(dT).

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