Resonance Raman Spectroscopy of 4-Thiothymidine and Oligodeoxynucleotides Containing This Base Both Free in Solution and Bound to the Restriction Endonuclease *Eco*RV†

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ABSTRACT: The resonance Raman spectra of 4-thiothymidine [^{4S}T] have been recorded (a) in the free deoxynucleoside form, (b) when incorporated into the single stranded oligodeoxynucleotide d(AG[^{4S}T]TC), and (c) within the double-stranded self-complementary dodecamer d(GACGA[^{4S}T]ATCGTC). Vibrational mode assignments of almost all the major Raman bands observed in each spectra have been made, mainly by comparison with the published assignments of related nucleosides and nucleotides. Differences between the spectra were observed, particularly when [^{4S}T] and d(AG[^{4S}T]TC) were compared to d(GACGA[^{4S}T]ATCGTC). This is explained in terms of the variations in structure between single- and double-stranded DNA. Good quality spectra were obtained at nucleotide/oligonucleotide concentrations of between 100 and 500 μM and this coupled with an apparatus that uses small volumes (100 μL) allowed measurement of the spectrum of d(GACGA[^{4S}T]ATCGTC) bound to the *Eco*RV endonuclease. This well characterised nuclease, for which crystal structures are available, recognizes d(GATAT) sequences. When this is replaced with d(GA[^{4S}T]ATC), a poor substrate results but turnover can be prevented during data accumulation by omission of the essential cation Mg^{2+}. Large shifts in several of the Raman bands were observed, and these have been related to the environment of the [^{4S}T] base in the protein-bound oligonucleotide as deduced from the crystal structure. The wavenumber for the C═S stretch vibration in free d(GACGA[^{4S}T]ATCGTC) has been used to calculate the strength of the Watson−Crick hydrogen bond between the sulphur atom in [^{4S}T] and the 6-NH_2 group on its partner dA. On binding to the enzyme, the shift in the wavenumber of the C═S stretch indicates this Watson−Crick hydrogen bond is weakened, in good agreement with X-ray structures. The advantage of using [^{4S}T] as a resonance Raman probe is that it absorbs at 340 nm, a wavelength where other nucleic acid and protein absorbance is minimal. Thus the spectra obtained are very simple and consist of signals that arise predominantly from the thiobase alone, and this facilitates data interpretation.

Over the last decade it has become apparent that B-DNA does not consist of a regular repeating structure, but rather that considerable deviations from idealized B-DNA geometry can be observed over dinucleotide steps or short sequences [for reviews, see Drew et al. (1988), Travers (1989), and Dickerson (1992)]. Furthermore, large distortions in "ideal" B-DNA parameters are often observed on binding to proteins, as shown by X-ray structures of oligodeoxynucleotides bound to, for example, repressor proteins (Otwinowski et al., 1988; Beamer & Pabo, 1992; Somers & Phillips, 1992), endonucleases (Weston et al., 1992; Winkler et al., 1993; Kostrewa & Winkler, 1995), and methylases (Klimasauskas et al., 1994). The appreciation of the structural variability of DNA has made methods for the determination of local oligodeoxynucleotide conformations very important. Crystallography gives extremely detailed information for both free DNA (Dickerson, 1992) and DNA bound to proteins [see above references and reviews Harrison (1991), and Pabo & Sauer (1992)]. Much useful information has also been obtained from NMR spectroscopy [for a review of DNA structural determination by NMR, see Feigon et al. (1992); for examples of NMR structures of protein-DNA complexes, see Billeter et al. (1993), Chuprina et al. (1993), and Omichinski et al. (1993)].

A number of other spectroscopic techniques can be valuable in the study of nucleic acids, even though they give less precise information than crystallography or NMR. Among these is Raman spectroscopy, which has been extensively used to investigate DNA structure in solution [see reviews by Peticolas et al. (1987), Thomas and Wang (1988), and Peticolas and Evertsz (1992)]. A number of useful "marker" bands indicative of the A, B, and Z forms

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have been established, and other bands are diagnostic of different sugar puckers (Thomas & Wang, 1988; Dijkstra et al., 1991). Certain bands can also be assigned to different bases (Benevides et al., 1991a,b). Using these features, a large amount of information about free DNA structures can be evaluated. This includes studies of the B ↔ Z transition (Wang et al., 1981; Thamann et al., 1981; Benevides et al., 1984) and switching between the B and A forms (Benevides et al., 1986; Nishimura et al., 1986). Raman spectra can be obtained from crystals, as well as solutions, allowing a comparison of DNA X-ray crystallographic structures with those seen in solution. Thus, Benevides et al., (1988) observed that d(CGCAAATTTGCG) exhibited a greater heterogeneity in sugar ring and phosphate backbones angles in the crystal, as compared to solution. Raman spectroscopy has also been used to probe the conformations of oligodeoxynucleotides bound to proteins. Examples have included the packaging of single-stranded DNA into phage ϕX174 capsids (Incardona et al., 1987; Benevides et al., 1991c), the interaction of single-stranded polynucleotides with the phage T4 gp32 protein (Otto et al., 1987), and the binding of the λ (Benevides et al., 1991a,b), cro (Evertz et al., 1991), and Ner (Benevides et al., 1994a) repressor proteins to their target DNA sites. Recently, Raman spectroscopy has been used to demonstrate that a mutation in the λ repressor that causes a change in specificity, also brings about a reorganization of the protein−DNA interface (Benevides et al., 1994b). Experiments with both free and *Eco*RI endonuclease-bound oligonucleotides have demonstrated that the *Eco*RI (GAATTC) recognition sequence is intrinsically distorted. This deviation, from ideal B-DNA structural parameters, is further increased on binding to the enzyme (Thomas et al., 1989).

One problem with Raman spectroscopy of DNA is that although marker bands can be reliably used to show the presence of a particular backbone or nucleoside conformation or to monitor the environment of the four bases, it is rare that a specific band can be assigned to an individual base, sugar, or phosphate. This difficulty is especially acute when a nucleic acid is bound to a protein. Here it would be expected that only a subset of the DNA components would experience conformational or environmental changes and, therefore, an alteration in their Raman peaks. Furthermore, unusual protein bound conformations could give rise to Raman bands well resolved from those typical of "ideal" B-DNA. Thus, although DNA conformational changes on binding to proteins are easily monitored by Raman spectroscopy and explainable in terms of alterations to phosphate torsional angles, sugar puckers, and base environment, it has been much harder to say precisely which phosphates, sugars, and bases are the ones changing. One way around this problem is to use resonance Raman spectroscopy (Nishimura et al., 1978). Here, the incident light wavelength matches that of an electronic absorbance band of the molecule, causing some of the Raman bands to become considerably more intense (Nishimura & Tsuboi, 1980). Unfortunately the UV (electronic) absorbances of the bases in nucleic acids and the aromatic amino acids in proteins have a high degree of overlap. Therefore it is difficult to specifically excite a particular base or aromatic amino acid, although by judicious choice of wavelength specific components, e.g., protein tyrosines and tryptophans in a protein−DNA complex, can be excited. However, the base analogues 4-thiouridine and 4-thiothymidine (Figure 1) both have UV absorbance maxima

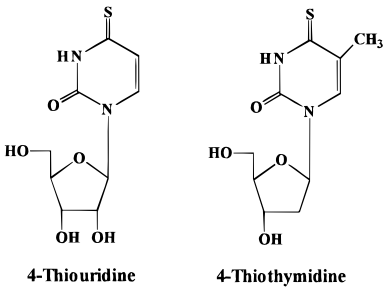

FIGURE 1: Structures of 4-thiouridine and 4-thiothymidine.

at 330−340 nm (Fox et al., 1959; Connolly & Newman, 1989; Nikiforov & Connolly, 1991), well removed from the four natural base constituents of DNA/RNA (260 nm) and also the aromatic amino acids in proteins (280 nm). Therefore, it should be possible to measure the resonance Raman spectra of these thiobases, using radiation in this spectral region, without enhancing the bands of other biological materials. The advantage of this is that the properties of a particular base in a nucleic acid, and any changes in its behavior on binding to a protein, can be examined with minimal interference and spectral overlap from other components. The disadvantage, when compared to conventional Raman spectroscopy, is that most of the bands (those arising from the nonresonant components) are not easily observed, and, thus, some information goes unrecorded.

The resonance Raman spectrum of 4-thiouridine has previously been measured (Nishimura et al., 1978), and many of the bands have been assigned to particular vibrational modes, especially by comparison to the Raman spectra of uridine derivatives (Nishimura et al., 1978, 1981; Tsuboi et al., 1987; Nishimura & Tsuboi, 1980). 4-Thiouridine is a natural constituent of some tRNAs (Favre et al., 1969), and the resonance Raman spectrum of this base in tRNA has been reported (Nishimura et al., 1976), demonstrating the use of this technique in establishing structure function relationships for polynucleotides. By analogy one would expect that 4-thiothymidine would also be an excellent resonance Raman probe, useful for the study of both free and protein-complexed DNA conformations. As far as we are aware, no resonance Raman spectra have been recorded for 4-thiothymidine, or this base incorporated into DNA, probably because (unlike 4-thiouridine) it does not occur naturally in nucleic acids. Recently, we (Nikiforov & Connolly, 1992a) and others (Coleman & Siedlecki, 1992) have described methods for the incorporation of 4-thiothymidine into synthetic oligodeoxynucleotides, using the phosphoramidite method, and protecting the sulfur atom with the β-cyanoethyl group. Other methods for synthesizing oligonucleotides containing this base have also been described (Nikiforov & Connolly, 1991; MacMillan & Verdine, 1991; Clivio et al., 1992; Xu et al., 1992). Oligodeoxynucleotides containing 4-thiothymidine have previously been used to study protein−DNA interactions with the *Eco*RV restriction endonuclease and modification methylase (Newman et al., 1990a,b) and to photoaffinity label the methylase (Nikiforov & Connolly, 1992b).

In this publication we extend the uses of oligonucleotides containing 4-thiothymidine by measuring the resonance Raman spectra of the free base and this thiobase incorporated into both single- and double-stranded oligodeoxynucleotides.

Most of the peaks seen in the spectra have been assigned to particular vibrational modes, and tentative explanations are given for the spectral differences observed between double- and single-stranded DNA. Finally, we have recorded the spectrum of d(GACGA[^{4S}T]ATCGTC) bound to the *Eco*RV endonuclease. This is a very well characterized protein [see reviews by Halford et al. (1993) and Vipond and Halford (1993)] which recognizes GAT↓ATC sequences and cuts them at the indicated site. The very similar GA[^{4S}T]ATC sequence is also recognized and slowly cleaved (Newman et al., 1990a,b). Crystal structures (Winkler 1992; Winkler et al., 1993; Kostrewa & Winkler, 1995) reveal large distortions to DNA substrates on binding to the protein that are centered on the central two TA bases of the GATATC recognition site. These perturbations are reflected in major changes to the resonance Raman spectrum of the thiothymidine containing oligonucleotide following binding.

MATERIALS AND METHODS

4-Thiothymidine was synthesized as described (Connolly & Newman, 1989). Oligodeoxynucleotides containing 4-thiothymidine were prepared using the phosphoramidite derivative of 4-(*S*-2-cyanoethyl)-thiothymidine (Nikiforov & Connolly, 1992) and purified by reverse-phase HPLC (Connolly, 1991). This phosphoramidite was either synthesized (Nikiforov & Connolly, 1992) or else purchased from Cruachem Ltd. (Glasgow, U.K.). The purification of the *Eco*RV endonuclease from overproducing strains has been published (D'Arcy et al., 1985; Luke et al., 1987).

Resonance Raman spectra were recorded using a Coherent Innova 90 K argon ion laser operating at 363.8 nm (10−50 mW output at the excitation source). Laser plasma lines were removed from the beam by the use of a Pellin-Broca dispersing prism. Raman scattered light was collected in the back-scattering mode (i.e., at 180° to the incident laser beam) with a Spex Triplemate spectrometer fitted with a 2400 g/mm UV blazed holographic grating in the final dispersion stage. Detection of radiation was with a Princeton Instruments liquid nitrogen cooled CCD camera. Spectra were calibrated to an accuracy of ±1.5 cm^{-1} using the ethanol, carbon tetrachloride, toluene, and acetone Raman peaks [wavenumbers taken from Schrader (1989)]. Spectra were recorded, over the range 500−1700 cm^{-1}, with an integration time of 90 s and a spectral slit width of 100 μm, from 100 μL samples in a rotating quartz cuvette (diameter 10 mm, height 3 mm) at 25 °C. All samples (except for the stock solutions of *Eco*RV endonuclease) were deoxygenated by bubbling argon through them prior to recording spectra. With 4-thiothymidine spectra were recorded in either H_2O, D_2O, buffer A (50 mM Hepes pH 7.5, 130 mM NaCl, 1 mM EDTA in H_2O) or buffer B (10 mM KH_2PO_4, pH 7.5, 100 mM NaCl in H_2O) at concentrations of 0.45 mM. The laser power dependence of the resonance Raman spectrum of 4-thiothymidine was investigated using conditions identical to those above (in H_2O only) at various laser powers of between 3 and 25 mW. The areas of the peaks at 710 and 1156 cm^{-1} were determined and plotted against laser power. The resonance Raman spectra of d(AG[^{4S}T]TC) and d(GACGA[^{4S}T]ATCGTC) were recorded in either buffer A or B at concentrations of between 0.4 and 0.5 mM. The resonance Raman spectrum of d(GACGA[^{4S}T]ATCGTC) bound to the *Eco*RV endonuclease was recorded in buffer A or B with 0.1 mM oligonucleotide and 0.11 mM endonuclease. The Raman spectrum of the endonuclease was also recorded under identical conditions but in the absence of oligonucleotide. In all cases at least 20 spectra were recorded per experiment to improve signal to noise, and the accumulated spectra were summed and peaks due to cosmic rays removed using CCD Spectrometric Analysis Software (Princeton Instruments). Any background fluorescence was removed by the subtraction of a polynomial curve fitted to the baseline. The peak fitting was carried out using the program, Raman, developed by the Central Laser Facility at the Rutherford Appleton Laboratory (Matousek et al., 1993; for further information about this program contact A.W.P.). The final assignments are accurate to ±1.5 cm^{-1}. Each experiment was repeated a minimum of five times. In some cases, to check for photodecomposition while recording the spectra, samples were analyzed for [^{4S}T] content before and after measurement by reverse-phase HPLC (Connolly & Newman, 1989; Newman et al., 1990a; Connolly, 1991).

The IR spectrum of 4-thiothymidine was recorded using a Nicolet 60 SX spectrometer. About 1 mg of the deoxynucleoside was dissolved in a small amount of acetonitrile and spread, as a thin film, onto an NaCl plate. The acetonitrile was removed in a vacuum desiccator, and 32 scans, between the wavenumbers 600 and 1800 cm^{-1}, were recorded and summed.

RESULTS AND DISCUSSION

Measurement of Resonance Raman Spectra of 4-Thiothymidine and Oligonucleotides Containing This Base. It proved relatively straightforward to record the resonance Raman spectra of 4-thiothymidine and several oligonucleotides containing the base, at concentrations of between 100 and 500 μM using 363.8 nm irradiation. A potential concern was photodecomposition of the thiobase which is known to be photoreactive (Favre, 1990). On illumination, 4-thiopyrimidines can be photooxidized, yielding pyrimidines, or undergo photoaddition, to give adducts at the C-6 position. Oligonucleotides containing 4-thiothymidine can also undergo photo-cross-linking to proteins (Nikiforov & Connolly, 1992b). We observed some photodecomposition during the course of resonance Raman measurements. Under the typical measurement conditions, outlined in the Materials and Methods, about 5% degradation of [^{4S}T] and d(AG[^{4S}T]TC) was observed with the major product being T (or an oligonucleotide containing this base). Much less decomposition was noted with the double-stranded 12-mers. Deoxygenation, by bubbling argon through all the solutions, greatly reduced photodecomposition. This is not unexpected as photooxidation is one of the main photochemical reactions of 4-thiopyrimidines. The use of pulsed excimer/dye lasers (363 nm radiation, 10 ns pulse width, ≈0.5 mJ at sample) caused greater photodecomposition and gave poorer quality spectra. None of the photoproducts had strong absorbance at 340−360 nm, and thus degradation simply reduces the intensity of the spectra and does not lead to new peaks. A persistent problem was a fluorescence background. This could be reduced, but never completely eliminated, by extensive purification of the oligonucleotides and the use of best quality reagents. The residual fluorescence background was therefore subtracted from the measured spectra. Finally, all spectra showed only a relatively few, well resolved peaks due to the use of the resonance mode and specific activation

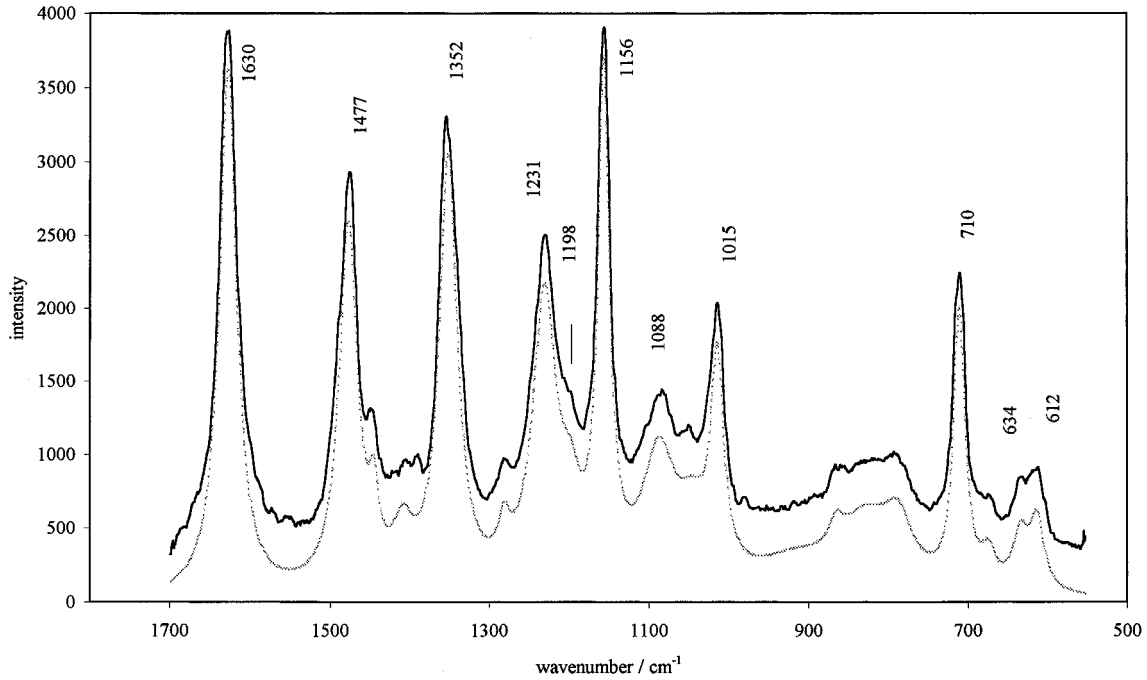

FIGURE 2: Resonance Raman spectrum of [^{4S}T], between 500 and 1700 cm^{-1}. This spectrum was recorded in H_2O using 363.8 nm excitation. The concentration of [^{4S}T] was 0.45 mM. The solid line gives the spectrum observed following subtraction of the fluorescent background. The lower dotted line, slightly vertically offset for clarity, is the fitted spectrum. The wavenumber values shown for the most prominent peaks are taken from this fit.

Table 1: Positions of the Peaks Seen in the Resonance Raman Spectra of 4-Thiouridine (Nishimura et al., 1976, 1978) and 4-Thiothymidine, d(AG[^{4S}T]TC), d(GACGA[^{4S}T]ATCGTC), and d(GACGA[^{4S}T]ATCGTC) Bound to the *Eco*RV Endonuclease[a]

vibrational mode	4-thiouridine	4-thiothymidine	d(AG[^{4S}T]TC)	d(GACGA[^{4S}T]-ATCGTC)	d(GACGA[^{4ST}]ATCGTC) bound to *Eco*RV
ring stretch ν_b^6 (C5═C6)	1619	1630	1632	1632	1622
ring stretch ν_a^6	1482	1477	1474	1471	1472
ring stretch $\nu_{\delta a}^6$ coupled with N1−C1′ stretch	1372	1352	1353	1398	1349
C5−methyl stretch		1352	1353	1356	1349
Kekulé Kk	1243	1231	1230	1239 (weak)	1236
ring breath Br^6 coupled with C═S stretch	1160	1156	1157	1157	1160
# ring stretch $\nu_{\delta b}^6$		1088	1100	1095	Identification difficult
# ring deformation Tr		1015	1015	1012	1008
unknown			790−830 (broad)	790−830 (broad)	830, 794 (broad)
C═S stretch coupled with ring breath Br^6	709	710	710	705	708
# ring deformation δ_a^6		634/612	633/612	633/613	611

[a] The assignment of individual peaks to particular vibrational modes is also given. All values are given as wavenumbers (cm^{-1}). The assignments of the peaks marked "#" must be considered tentative at present.

of the thiobase at 363.8 nm. No strong transitions were seen for the *Eco*RV endonuclease under the conditions used.

Assignment of the Resonance Raman Spectrum of 4-Thiothymidine. The resonance Raman spectrum of free 4-thiothymidine is shown in Figure 2. The spectrum shown was recorded in H_2O, but those in buffers A and B were essentially identical. Prior to interpretation it is necessary to assign individual bands in the spectrum to particular vibrational modes of the molecule. This is reasonably straightforward by analogy with reported assignments for the related compounds uracil, uridine, thymidine, and 4-thiouridine (Nishimura et al., 1976, 1978; Fodor et al., 1985; Tsuboi et al., 1987). The resonance Raman spectrum of 4-thiothymidine is similar to that of the well characterized 4-thiouridine and this provides a starting point. Six major bands are seen for 4-thiouridine at wavenumbers of 709, 1160, 1234, 1372, 1482, and 1619 cm^{-1} (Nishimura et al., 1976, 1978). In the spectrum of 4-thiothymidine, shown in Figure 2, five of the bands (those at 710, 1156, 1231, 1477, and 1628 cm^{-1}) occur at very similar wavenumbers allowing their immediate assignment. Thus, as summarized in Table 1, the bands at 709 and 1156 cm^{-1} can be assigned to the strongly coupled C═S stretching mode and the ring breathing vibration Br^6. The bands at 1231, 1477, and 1628 are attributable to Kekulé (Kk), ν_a^6, and ν_b^6 (C5═C6 stretch) ring vibrational modes, respectively (Nishimura et al., 1978; Tsuboi et al., 1987; the latter reference discusses the nature of these ring vibrational modes). It should be noted that the Kk vibration at 1231 cm^{-1} is very near a weak band, just visible as a shoulder, at 1198 cm^{-1}. This will become important when the spectra of the oligonucleotides are discussed. The main difference between the 4-thiouridine and 4-thiothymidine spectra is the replacement of the weak peak at 1372 cm^{-1}, in the former, with a very much more intense peak at 1352 cm^{-1} in the latter. In the 4-thiouridine spectrum, the peak at 1372 cm^{-1} is the most difficult to assign. By comparison with uracil and uridine derivatives, it has been tentatively attributed to a blend of the N1−C1′ stretch and the ring stretch $\nu_{\delta a}^6$ (Tsuboi et al., 1987). We believe that the strong peak at 1352 cm^{-1} for 4-thiothymidine

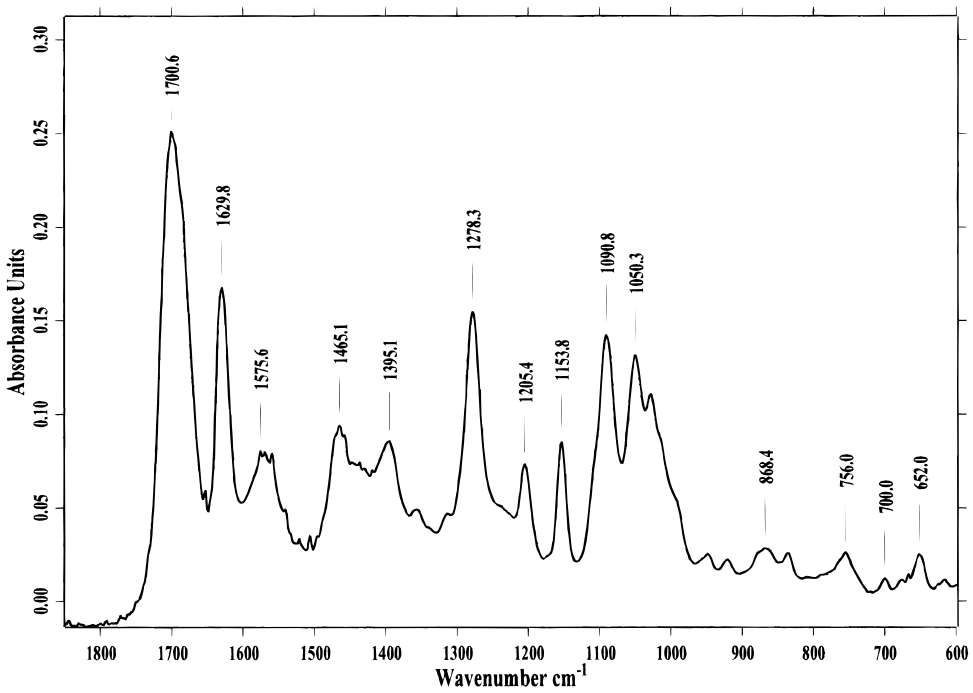

FIGURE 3: Infrared spectrum of [^{4S}T], between 600 and 1850 cm^{-1}. This spectrum was recorded from ≈1 mg of [^{4S}T] spread on an NaCl plate.

can be assigned to a combination of this N1−C1′ stretch coupled with the $\nu_{\delta a}{}^6$ stretch together with the C5−Me stretch on the basis of the following: (i) the 5-methyl group is the only difference between the two thiobases; (ii) an extra, strong peak at 1379 cm^{-1} is also a prominent difference between the resonance Raman spectra of dTMP and dUMP (Fodor et al., 1985); (iii) a peak at 1376 cm^{-1} in the normal mode Raman spectra of oligodeoxynucleotides has been assigned to the thymidine C5 methyl group (Benevides et al., 1991b). Further investigation of this 1352 cm^{-1} peak was conducted by recording the resonance Raman spectrum of 4-thiothymidine in D_2O (not shown). This causes the replacement of the exchangeable N3 proton with a deuteron. The 1352 cm^{-1} resonance disappeared and was replaced by two new peaks at 1330 and 1366 cm^{-1}. The 1352 cm^{-1} peak seen in H_2O was attributed to the two vibrations N1−C1′ plus $\nu_{\delta a}{}^6$ stretch and C5−Me stretch being degenerate and thus producing a single band. Presumably, deuteration affects the coupling of these two modes causing the 1352 cm^{-1} peak to be split into the two components. This is supported by the finding that the total relative areas of the 1330 and 1366 cm^{-1} peaks in D_2O approximate to the relative area of the 1352 cm^{-1} peak in H_2O. The transitions seen for 4-thiothymidine at 612, 634, 1015, and 1088 cm^{-1} are difficult to assign unambiguously as they have no strong counterparts in the resonance Raman spectra of related pyrimidines (Fodor et al., 1985). A peak in the 612/634 region was observed for 4-thiouridine (Nishimura et al., 1976) but not discussed. The 1015 and 1088 peaks are, respectively, near to the Tr ring deformation and the $\nu_{\delta b}{}^6$ ring stretch of uridine (Tsuboi et al., 1987), observed under nonresonant Raman conditions, and have been given these assignments. The attribution of the more intense 1015 cm^{-1} band to the symmetric Tr deformation and the less intense 1088 cm^{-1} to the more asymmetric $\nu_{\delta b}{}^6$ mode fits with the fact that symmetric modes are generally more intense than asymmetric in Raman spectroscopy. The 612/634 cm^{-1} peaks are near the $\delta_a{}^6$ ring deformation, and so one of these two resonances probably corresponds to this mode. Nevertheless, these last assignments should be considered tentative at present.

We have also recorded the infrared spectrum of 4-thiothymidine, and this is illustrated in Figure 3. Several of the bands seen in the resonance Raman spectrum (those at 1088, 1156, 1477, and 1630) are prominent in the IR, to within a few wavenumbers. A notable, but expected, absence is the lack of a C═S stretch peak at around 710 cm^{-1}. The IR also contains a very prominent peak at 1700 cm^{-1} corresponding to the C_2═O stretch that is not present in the resonance Raman spectrum. This is frequently encountered in resonance Raman spectroscopy because vibrational modes associated with the electronically excited chromophore are preferentially enhanced and/or there is a change in the molecular geometry that mirrors a normal vibrational mode of the ground state molecule (Nishimura & Tsuboi, 1980). The 260 nm absorbance band of uracil (and the corresponding 340 nm band of 4-thiopyrimidines) involves a $\pi\pi^*$ electronic transition associated mainly with the 5C═6C−4C═O(S) section of the molecule and does not involve the 2C═O element. This explains why no resonance Raman transition corresponding to a C_2═O vibration is seen in Figure 2 or for any pyrimidine derivative using 260 (340) nm wavelengths.

To confirm that the Raman spectrum is of ground state 4-thiothymidine and not due to an excited state, the dependence of Raman band intensities at 710 and 1156 cm^{-1} as a function of laser power was investigated, and the results obtained are shown in Figure 4. If an electronically excited state is being observed, then the Raman band intensities are expected to be proportional to (laser power)^2. This is caused by absorbance of an initial photon, to give an electronically excited state, followed by a second photon being scattered by the excited molecule to produce the Raman spectrum. If the ground state is responsible, only the scattered photon takes part, giving Raman intensity directly proportional to

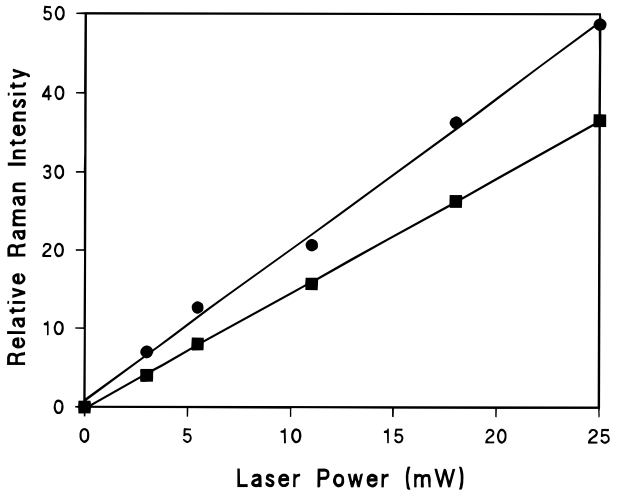

FIGURE 4: Power dependence of the resonance Raman intensities of the 710 cm^{-1} (■) and the 1156 cm^{-1} (●) peaks of 4-thiothymidine. An excitation wavelength of 363.8 nm was used, and the concentration of [^{4S}T] was 0.45 mM. The spectra were recorded in water.

laser power. The latter situation was seen which clearly confirms that the ground state was observed.

Resonance Raman Spectra of 4-Thiothymidine in Oligodeoxynucleotides. The resonance Raman spectrum of the self-complementary double-stranded dodecamer d(GACGA-[^{4S}T]ATCGTC) is shown in Figure 5. The bands together with their assignments are documented in Table 1. This table also contains the resonance Raman bands seen for the single stranded pentamer d(AG[^{4S}T]TC) (the spectra of the pentamer is not shown but was of similar quality to that given in figure 5). These spectra were obtained in buffer A, but B gave similar results. In general the spectra of free [^{4S}T] and the pentamer are similar but differ in several respects from the dodecamer. However, a full interpretation of all the bands, and the shifts in bands that occur when 4-thiothymidine is incorporated into double stranded oligonucleotides, is not yet possible. This discussion is therefore limited to the more easily interpreted features.

One of the more interesting effects is the position of the C═S stretch, which occurs at 710 cm^{-1} for free [^{4S}T] and single-stranded d(AG[^{4S}T]TC), but is shifted to 705 cm^{-1} for double-stranded d(GACGA[^{4S}T]ATCGTC). This is undoubtedly due to the formation of a Watson−Crick hydrogen bond between the sulfur atom in [^{4S}T] and the 6-NH_2 group of its partner dA base. This is supported by the shift in the position of this peak back toward 705 cm^{-1} as d(GACGA[^{4S}T]ATCGTC) is heated and the double strands are melted (data not shown). It has previously been observed, using resonance Raman spectroscopy, that the C_4═O stretch (which occurs at 1650 cm^{-1}) in T/dA base pairs increases in frequency with a decrease in hydrogen bonding strength (Toyama et al., 1991). It is possible to estimate the energy of this Watson−Crick hydrogen bond using two methods. The first relies on an estimate that there is a linear relationship between bond energy and wavenumber shift as long as the wavenumber shift is small in relation to the overall bond energy (Badger & Bauer, 1937). Thus with d(GACGA[^{4S}T]ATCGTC a 5 cm^{-1} shift relative to [^{4S}T] or d(AG[^{4S}T]TC) is observed. Taking a value of 600 kJ mol^{-1} for the C═S bond (Gans, 1971), this gives a change in ΔH of

$$\Delta\Delta H = (5/710) \times 600 = 4.2 \text{ kJ mol}^{-1}$$

A second method uses the empirical correlation between the change in bond force constant, F, and ΔH (Gans, 1971). Here simple harmonic motion is assumed for the vibration and the change in force constant is calculated from the equation for simple harmonic motion:

$$[\text{frequency}]^2 = \text{force constant/reduced mass}$$

$$\text{ratio of force constants} = (710)^2/(705)^2 = 1.014$$

Therefore there is a 1.4% decrease in the force constant. The slope of a tangent drawn to the nonlinear relationship between F and ΔH at a value of ΔH of 600 kJ mol^{-1} (the C═S bond strength) gives

$$\Delta H = 0.61F$$

The decrease in ΔH is therefore, $0.61 \times 1.4\% = 0.85\%$

$$\Delta\Delta H = 0.85\% \times 600 = 5.1 \text{ kJ mol}^{-1}$$

This second method of calculation has been previously used for chymotrypsin acylenzymes (White & Wharton, 1990). These two approaches do not necessarily yield exact values, but the fact that they give similar results is encouraging. Also they are of similar magnitude to the strength of Watson−Crick hydrogen bonds in DNA duplexes in water, which are reported to lie between 3.3 and 6.7 kJ mol^{-1}, with an average of about 4 kJ mol^{-1} (Crothers & Zimm, 1964; Freier et al., 1986). With 4-thiothymidine, the usual oxygen (from the 4-thymidine position)/amino (from the 6-deoxyadenosine position) Watson−Crick hydrogen bond is replaced by one involving a sulfur. However, the almost identical T_m values seen with d(GACGA[^{4S}T]ATCGTC) and the parent d(GACGATATCGTC) (Connolly & Newman, 1988; Newman et al., 1990a) suggest that these two types of hydrogen bonds will be of about equal strength.

The peak at 1352 cm^{-1}, seen in the spectrum of [^{4S}T], and assigned to a combination of the $\nu_{\delta a}{}^6$ ring stretch and the N1−C1′ stretch together with the C5−methyl stretch, is also present, at about the same position, with d(AG[^{4S}T]TC). However, this peak is split in the double-stranded dodecamer giving two new bands at 1356 and 1398 cm^{-1}. This is, in a similar manner to above, most probably due to the N3 hydrogen atom of [^{4S}T] taking part in Watson−Crick hydrogen bonding to the N1 ring nitrogen of its partner dA. The extra mass connected to the pyrimidine ring is likely to perturb the $\nu_{\delta a}{}^6$/N1−C1′ stretching modes causing a separation from the C5−methyl stretch and resulting in the observed splitting of the bands. A similar effect was observed with [^{4S}T] in D_2O. We assign the relatively unchanged 1356 cm^{-1} band to the C5−methyl stretch as the location of this resonance varies only slightly between free deoxynucleosides and DNA. The origins of the large shift in the $\nu_{\delta a}{}^6$/N1−C1′ resonance, of almost 50 wavenumbers, remain obscure. It is probably due to a combination of the Watson−Crick interactions mentioned above and possibly the constraints imposed on the N1−C1′ bond angle in double-stranded DNA.

The peak seen with the duplex at 1095 cm^{-1} is near that at 1088 in [^{4S}T], which had previously been assigned to the $\nu_{\delta b}{}^6$ ring stretch. It is likely that the increase in wavenumber is brought about by a tightening of the pyrimidine ring due

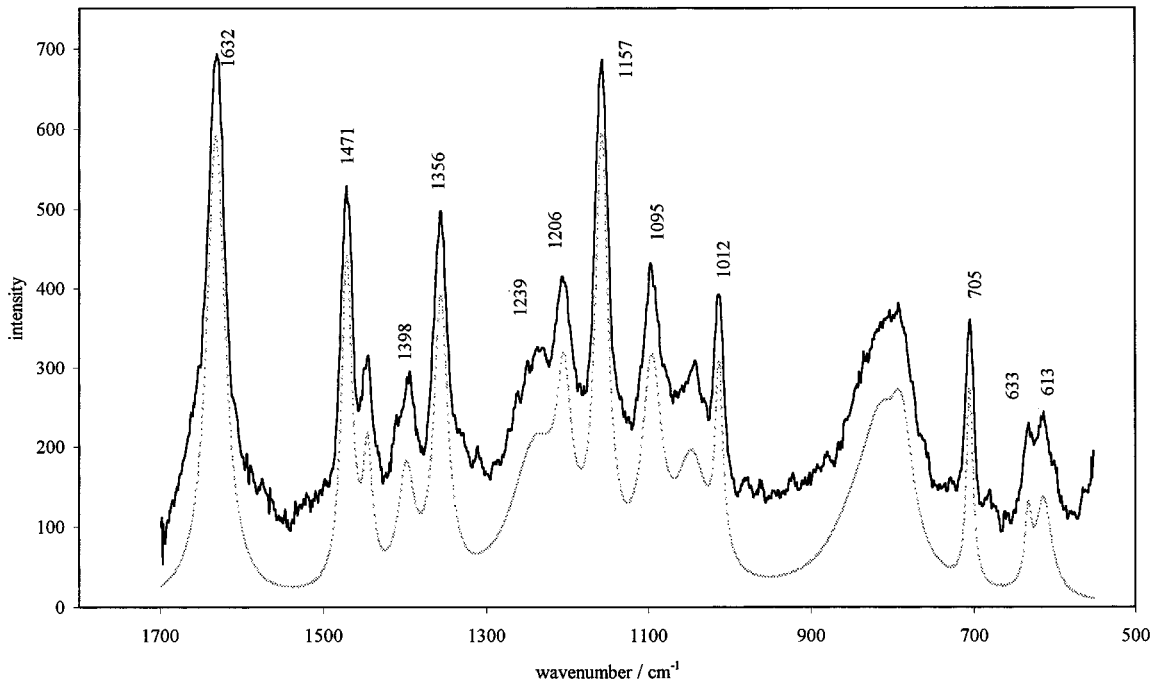

FIGURE 5: Resonance Raman spectrum (363.8 nm excitation) of d(GACGA[^{4S}T]ATCGTC) between 500 and 1700 cm^{-1}. The spectrum was recorded at an oligonucleotide concentration of 0.5 mM in 50 mM Hepes, pH 7.5, 130 mM NaCl, and 1 mM EDTA (buffer A). The solid line gives the spectrum observed following subtraction of the fluorescent background. The lower dotted line, slightly vertically offset for clarity, is the fitted spectrum. The wavenumber values shown for the most prominent peaks are taken from this fit.

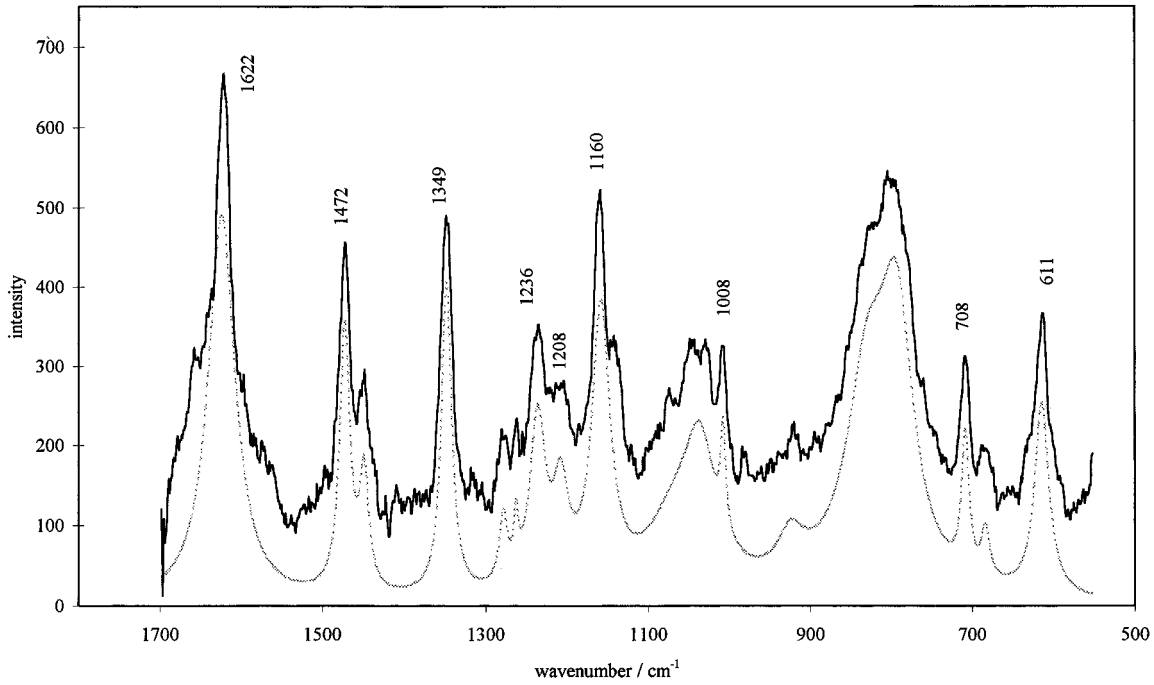

FIGURE 6: Resonance Raman spectrum (363.8 nm excitation) of a mixture of d(GACGA[^{4S}T]ATCGTC) (0.1 mM) and *Eco*RV endonuclease (0.11 mM) between 500 and 1700 cm^{-1}, recorded in 50 mM Hepes, pH 7.5, 130 mM NaCl, and 1 mM EDTA (buffer A). The solid line gives the spectrum observed following subtraction of the fluorescent background. The lower dotted line, slightly vertically offset for clarity, is the fitted spectrum. The wavenumber values shown for the most prominent peaks are taken from this fit.

to incorporation into a polymeric oligonucleotide. The broad feature between 790 and 840 cm^{-1} is of unknown origin but completely reproducible. It is a very prominent in oligonucleotides (both single- and double-stranded) as can been seen in Figure 5 for d(GACGA[^{4S}T]ATCGTC) and also in Figure 6 for this oligonucleotide bound to the *Eco*RV endonuclease. An oligonucleotide, d(GACC[^{4S}T]ATAGGTC, used as a control also shows this feature (Figure 7). It can also been seen as a very much weaker feature with [^{4S}T] (Figure 2). It is unlikely that this broad peak arises from errors in the fluorescence subtraction as it is clearly visible in the uncorrected spectra. One possibility is a contaminant in either the buffers or the oligonucleotide. However high quality reagents were used for the buffers and the oligonucleotides were extensively purified. A less likely explanation is that, with the oligonucleotides, the peak is due to poorly resolved backbone O−P−O torsional vibrations. These occur in this region as a very characteristic marker of B-DNA (Thomas & Wang, 1988; Peticolas et al., 1987). However, backbone vibrations cannot be resonance en-

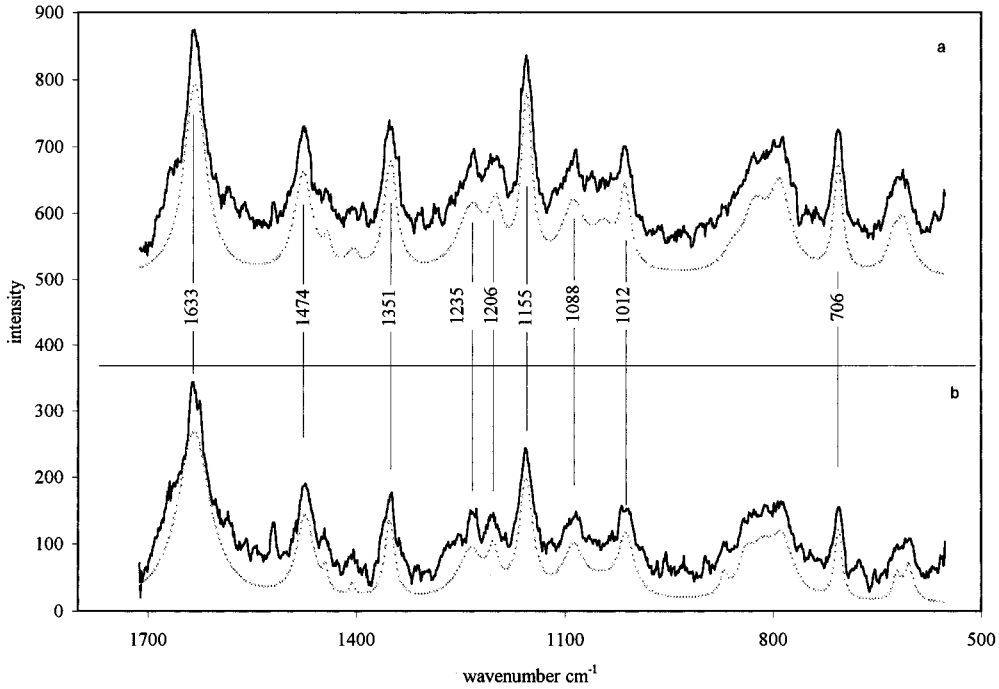

FIGURE 7: (a) Resonance Raman spectrum (363.8 nm excitation) of d(GACC[^{4S}T]ATAGTC) between 500 and 1700 cm^{-1}. The spectrum was recorded at an oligonucleotide concentration of 0.1 mM in 50 mM Hepes, pH 7.5, 130 mM NaCl, and 1 mM EDTA (buffer A). (b) As in panel a, but in the additional presence of 0.11 mM *Eco*RV restriction endonuclease. The solid line gives the spectrum observed following subtraction of the fluorescent background. The lower dotted line, slightly vertically offset for clarity, is the fitted spectrum. The wavenumber values shown for the most prominent peaks are taken from this fit.

hanced. Even though there are 11 phosphates in the dodecamer and backbone vibrations are normally intense bands, it would seem unlikely that a nonresonant band would be so prominent. The final major change in the dodecamer spectra is in the region of the Kk vibrational mode. With [^{4S}T] and d(AG[^{4S}T]TC) this occurs at 1231 cm^{-1}, near a much weaker shoulder at 1198 cm^{-1}. With d(GACGA[^{4S}T]-ATCGTC) the Kk band is shifted to 1239 cm^{-1} but is much diminished in intensity. Thus the 1198 cm^{-1} band, which with this dodecamer is shifted to 1206 cm^{-1}, dominates the spectra. The 1239 cm^{-1} band almost certainly corresponds to the 1238 cm^{-1} transition observed for thymidine in nonresonance Raman spectroscopy. This is the most hypochromic band in calf thymus DNA, and its intensity increases dramatically as the base unstacks (Benevides et al., 1991c). Thus the 4-thiothymidine band at 1231 cm^{-1} (free deoxynucleoside)/1239 cm^{-1} (dodecamer) is expected to be much reduced in intensity in the double-stranded dodecamer, due to base stacking. An extra peak at 1242 cm^{-1} is also seen for the double-stranded 12-mer, which is absent in 4-thiothymidine. This transition occurs at 1254 cm^{-1} with the pentamer.

Resonance Raman Spectra of Oligodeoxynucleotides Bound to the EcoRV Endonuclease. The spectrum of d(GACGA-[^{4S}T]ATCGTC) bound to the restriction endonuclease *Eco*RV is shown in figure 6. This spectra was recorded in buffer A in the absence of Mg^{2+} to prevent turnover. Similar spectra were obtained using buffer B. However, in the absence of this cation the enzyme still binds to DNA (Taylor et al., 1991). The K_d values that are observed for GATATC containing oligonucleotides, 12 base pairs in length, are about 1 μM (Taylor et al., 1991; Erskine and Halford, personal communication). In fact the endonuclease binds to all 12-mers with a similar K_d (Erskine and Halford, personal communication) as, in the absence of Mg^{2+}, there is no binding discrimination between sequences (Taylor et al., 1991). We have determined the K_d value for d(GACGA-[^{4S}T]ATCGTC) and obtain a value in the region of 1 μM (not shown), in good agreement with the above studies. This means that, at the concentration of endonuclease (110 μM) and oligonucleotides (100 μM) used in Figure 4, all the DNA will be enzyme bound, and free DNA levels will be vanishingly low. We have also previously shown that d(GACGA[^{4S}T]ATCGTC) is a substrate for the endonuclease but is turned over much more slowly than d(GAC-GATATCGTC) (Newman et al., 1990b). In agreement with the above K_d values, the K_m's were similar, 3.8 μM for the parent and 0.5 μM for the thiothymidine-containing oligonucleotide. However, the k_{cat} values were 6.9 and 0.016 min^{-1}, respectively.

As with the above results, we have limited our discussion to the most prominent and easily interpreted shifts in peaks, seen when d(GACGATA[^{4S}T]CGTC) binds to the enzyme. One striking effect is a 3 cm^{-1} shift for the C═S peak from 705 to 708 cm^{-1}. Thus on binding the enzyme this peak shifts its position from a location characteristic of double-stranded DNA (i.e., the C═S function being hydrogen bonded to its partner dA) toward a position indicative of single-stranded DNA (no Watson−Crick hydrogen bonding). However, the shift does not go all the way to the 710 cm^{-1} seen with free [^{4S}T] and the pentamer. Thus it is consistent with the weakening, but not the complete rupture, of the Watson−Crick hydrogen bond between the C═S group an the 6-amino function of its dA partner. This shift is highly reproducible, and it has been observed on every occasion that we have recorded such spectra. The crystal structure of the *Eco*RV endonuclease bound to several cognate oligodeoxynucleotide has been solved (Winkler, 1992; Winkler et al., 1993; Kostrewa & Winkler, 1994). Examination of this structure shows that the central T/dA base pairs in

the GATATC recognition sequence (i.e., the T residues that have been substituted with [^{4S}T]) are extremely distorted. A 50° kink is observed in this location and the major groove becomes narrower and deeper, whereas the minor groove becomes shallower. Base stacking between the T and dA base pairs on the same strand is essentially nonexistent. Furthermore, a four center arrangement that comprises the two 4-keto oxygen atoms and the two 6-amino groups from both the T and dA residues, which make up the central T/dA base pairs, is seen. All four groups are in close proximity, and it has been suggested that the center is stabilized by the partial negative and positive polarization of the keto and amino groups, respectively. Additionally, no hydrogen bonds, or any other interactions, are made between these bases, and the protein and the bases are also well shielded from solvent. One effect that the buckling of these central bases would be expected to have is to weaken the normal Watson−Crick hydrogen bonds between the T and dA base pairs. The 3 cm^{-1} upfield shift of the C═S peak seen in the Raman spectrum is completely consistent with this idea and in full agreement with the crystal structures. The rupture of T/dA Watson−Crick interactions upon the binding of DNA to the SPKK protein has been observed by resonance Raman spectroscopy (Takeuchi & Sasomori, 1994). In this case the thymidine C_4═O stretch was shifted 5 cm^{-1} upfield.

In the above discussion we noted that the $\nu_{\delta a}{}^6$/N1−C1′ and C5−Me vibrations were well separated in the double-stranded oligonucleotide, in contrast to free [^{4S}T] or single-stranded species. It was suggested that base pairing might have been partially responsible for the large positional shift of the $\nu_{\delta a}{}^6$/N1−C1′ transition. It is therefore gratifying to observe that these two bands once again occur at the same position (1349 cm^{-1}) when the 12-mer is bound to the enzyme. This is clearly consistent with weakening of base pairing in the endonuclease/DNA complex and the consequent movement of the $\nu_{\delta a}{}^6$/N1−C1′ vibration back to a value near that of [^{4S}T]. However, base stacking may also contribute as 4-thiothymidine is not, or weakly stacked, in [^{4S}T], single-stranded and bound oligonucleotides but highly stacked in the free double-stranded 12-mer. The resulting new peak, at 1349 cm^{-1}, includes a contribution from the C5−Me stretching vibration. This occurs at 1356 cm^{-1} in the unbound 12-mer, and so the C5−Me stretch is shifted by 7 cm^{-1} when the 12-mer binds to the endonuclease. The C5−Me vibration in thymidine is known to be environment sensitive and, for example, increases strongly in intensity on binding to the λ repressor (Benevides et al., 1991a,b). It was suggested that this arose from an increase in the hydrophobicity of the methyl group environment. As this peak is sometimes a mixture of two modes in our spectra, we are unable to draw any conclusions about intensity changes. However the 7 wavenumber shift in position must be due to the different surroundings that this CH_3 group finds itself in, following binding to the endonuclease. An examination of the crystal structures shows that no direct van der Waals contacts are formed between this methyl group and the protein. Nevertheless, the CH_3 group of this thymidine is in a loose hydrophobic pocket, which is formed from the hydrocarbon parts of the side chains of Thr106, Thr 186, and Asn 188 and is well shielded from solvent. This increase in hydrophobicity might contribute to the 7 cm^{-1} shift. However, other factors, such as the lessening of Watson−Crick interactions and the large decrease in stacking seen with this base, may also be responsible. The importance of this methyl group, for DNA recognition and cleavage by the endonuclease, has been demonstrated by both (1) base analogues, its replacement with dU gives poor substrates (Fliess et al., 1986, 1988; Mazarelli et al., 1989; Newman et al., 1990a,b), and (2) site-directed mutagenesis, altering the above amino acids interferes with catalysis (Thielking et al., 1991; Wenz et al., 1994).

In the above sections it was observed that the Kk vibration, occurring at 1231/1239 cm^{-1}, was very prominant for single-stranded [^{4S}T] and the pentamer but much reduced in intensity with the duplex. The nearby band at 1198/1206 cm^{-1} provides a convenient internal reference for the intensity of the Kk vibration. On binding of the 12-mer to the endonuclease, the Kk peak increases in intensity and becomes much more prominent than the 1206 cm^{-1} vibration (in this complex at 1208 cm^{-1}). In addition there is a slight positional shift to 1236 cm^{-1}. As discussed above, the intensity of the Kk resonance is extremely sensitive to base stacking and increases dramatically as stacking decreases. This result, therefore, is in full agreement with the crystal structure, which shows a complete lack of stacking of this pyrimidine base with those adjacent to it, when the oligonucleotide is bound to the *Eco*RV endonuclease. The shift in wavenumber is also, presumably, due to the highly perturbed environment of the base. We also note a large shift, by 10 cm^{-1}, in the position of the C5═C6 ($\nu_b{}^6$) ring stretch following binding of the dodecamer to the enzyme. At present we are unable to fully explain this but believe it relates to the strong coupling of this mode through the delocalisation of the π electrons with the C═S chromophore.

Finally, we have also measured the resonance Raman spectra of two further 4-thiothymidine containing oligonucleotides, d(GACGATA[^{4S}T]CGTC) and d(GACC[^{4S}T]-ATAGGTC). These were very similar to that observed for d(GACGA[^{4S}T]ATCGTC) as is shown in Figure 7 for d(GACC[^{4S}T]ATAGGTC). The signal to noise ratio of this spectrum is not as good as the others, but most of the peaks seen for d(GACC[^{4S}T]ATAGGTC) occur within 3−4 wavenumbers of those found with d(GACGA[^{4S}T]ATCGTC). A similar effect was observed with d(GACGATA[^{4S}T]CGTC) (not shown). Both of these new oligonucleotides bind to the endonuclease with a K_d value near 1 μM, but neither are substrates for the enzyme (Newman et al., 1990a). When the spectra of d(GACGATA[^{4S}T]CGTC) and d(GACC[^{4S}T]-ATAGGTC) were recorded in the presence of the *Eco*RV endonuclease, under conditions identical to those given above, there was, within experimental error, no change to the spectra as is illustrated in Figure 7 for d(GACC[^{4S}T]-ATAGGTC). Thus the simple binding of a 4-thiothymidine containing oligonucleotide to the endonuclease is, in itself, not sufficient to produce changes in the resonance Raman spectrum. For the three oligonucleotides used in this study, a substrate (even though catalysis is prevented while recording the spectrum by the exclusion of Mg^{2+}) is needed to bring about the spectral changes.

CONCLUSIONS

We have demonstrated that it is possible to measure the resonance Raman spectra of [^{4S}T] both as a free deoxynucleoside and when incorporated into oligodeoxynucleotides. Although the resonance Raman spectrum of the related

base 4-thiouridine, both free and in tRNA, has been measured previously, as far as we are aware this publication documents the first studies with 4-thiothymidine. Many of the peaks seen in its spectra have been assigned to particular vibrational modes by analogy with related compounds. While most of these assignments are reasonably secure, some are more tentative and require more work to fully elucidate. It has also proven possible to obtain spectra from d(GACGA[^{4S}T]-ATCGTC) bound to the *Eco*RV restriction endonuclease. These results allow comparisons to be made with the X-ray crystallographic data, and our results are in agreement with the likely mechanism of action of the enzyme. The crystal structures show that GATATC containing sequences are severely distorted on binding to the enzyme. As mentioned above, part of this distortion involves the central TA bases, which become very poorly stacked and have weakened base pairs. These bases are shielded from the solvent by the protein and the rest of the DNA and do not make any direct contacts to the protein. Another consequence of this distortion is to move the scissile phosphate toward the active site and simultaneously create a high affinity binding site for the essential cation Mg^{2+} (Taylor & Halford, 1989; Taylor et al., 1991; Vermote & Halford, 1992; Vermote et al., 1992). This allows metal binding and hence catalysis to take place. Although DNA sequences that lack GATATC sites bind efficiently to the endonuclease, they do so without any DNA distortion and so are not cleaved. The Raman spectra seen with enzyme-complexed d(GACGA[^{4S}T]ATCGTC) are clearly consistent with DNA distortion on binding. There are large shifts in several of the peaks associated with the [^{4S}T] base, and its spectrum is more akin to single-stranded, rather than duplex, DNA. This is expected from a weakly stacked and poorly base paired deoxynucleoside. This oligonucleotide is a substrate, albeit a poor one, and so would be expected to be distorted on binding to the endonuclease. The minimal spectral changes seen when d(GACGATA[^{4S}T]CGTC and d(GACC[^{4S}T]ATAGGTC), both nonsubstrates, bind to the enzyme are again in agreement with the idea that noncognate sequences bind with no distortion. It is thus clear that the 4-thiothymidine base is potentially a very powerful Raman probe, allowing the monitoring of the environment of a single base within an oligonucleotide using specific irradiation at 363 nm. The method described in this paper should be an excellent complement to normal Raman spectroscopy. Currently the use of 4-thiothymidine is limited only by an incomplete understanding of its Raman spectral properties and the lack of a full appreciation of which bonds contribute to the observed vibrational transitions. We hope that the demonstration of how simple it is to record resonance Raman spectra of this base and the potential usefulness of the method will encourage research in this area.

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