The solution conformations of a mutant *trp* operator determined by n.m.r. spectroscopy

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The principal conformational features of the mutant *trp* operator [d(CGTACTGATTAATCAGTACG)₂] have been determined by n.m.r. at different temperatures. The sugar puckers were determined from *J*-resolved spectroscopy and high-resolution homonuclear Hartmann–Hahn spectroscopic (HOHAHA) experiments. Extensive one-dimensional nuclear-Overhauser-enhancement (NOE) data sets were acquired at 25 °C using irradiation times of 50, 100, 200, 300 and 500 ms to generate sufficient NOE information to determine the individual nucleotide conformations, and place limits on the local helical parameters, using multi-spin least-squares fitting and searching in conformation space with the program NUCFIT [Lane (1990) *Biochim. Biophys. Acta* **1049**, 189–204]. The conformations of the nucleotides are well determined, and show significant sequence-dependent variation. Pyrimidine residues on average have a wider range of sugar conformations and smaller glycosidic torsion angles than purine residues. The helical parameters are in general less well determined, though clear evidence was obtained for sequence-dependent variation of the helical twist. The overall mean fractional deviation of the calculated from the observed NOEs was 0.108. The conformations of the base-pairs TAAT are temperature-dependent [Lane (1989) *Biochem. J.* **259**, 715–724]. NOESY spectra were recorded at 10, 25 and 40 °C, using mixing times inversely proportional to the overall tumbling time to allow changes in the conformation to be described. A more detailed analysis was made using one-dimensional NOEs collected for nucleotides involved in the conformational transitions. There are significant temperature-dependent changes in the conformations of the central base-pairs from T⁹ to T¹³ with the largest changes in the glycosidic torsion angle occurring for A¹¹ and A¹² (up to 30°). The orientation of the base-pairs T⁹–A¹²:T¹⁰–A¹¹ also changes, with an increase in the base-pair roll and an unwinding of the helix as the temperature is increased. The conformational changes are qualitatively similar to those observed in a related sequence (Lefèvre, Lane & Jardetzky (1988) *Biochemistry* **27**, 1086–1094]. The conformation is also similar to the wild-type sequence and to that observed in the crystal state in the complex with the *trp* holorepressor. The similarity suggests that the mutation produces a poorer operator by virtue of removal of essential functional groups within the major groove.

INTRODUCTION

The structures of numerous oligodeoxynucleotides have been determined in solution using n.m.r. data. The majority of these molecules have sequences corresponding to biologically interesting sites, such as operators (Scheek *et al.*, 1984; Weiss *et al.*, 1984; Lefèvre *et al.*, 1985, 1987; Grütter *et al.*, 1988), promoter Pribnow boxes (Lefèvre *et al.*, 1987, 1988; Nerdal *et al.*, 1988), and restriction-endonuclease sites (Chou *et al.*, 1984; Nilges *et al.*, 1987*a,b*; Chary *et al.*, 1988; Banks *et al.*, 1989; Nerdal *et al.*, 1989). In most instances, the overall conformations have been in the B-family, though clear sequence-dependent variations of the structure are observed, broadly in agreement with the rules of Calladine (1982) and Dickerson (1983) (Lefèvre *et al.*, 1987; Nilges *et al.*, 1987*a,b*). One of the goals of these studies is to find the conformational features that may be involved in recognition by the relevant protein. Recently, Reid and co-workers (Nerdal *et al.*, 1988, 1989; Banks *et al.*, 1989) have used distance geometry to generate structures, compatible with n.m.r. data, that show extensive kinking and underwinding of the double helix. These features could potentially be used in the recognition process by the cognate proteins.

The question remains, however, as to whether the observed conformational features, if they are indeed involved in recognition, differ in some way from other sequences, which can only be ascertained by a comparison of several structures. One particularly relevant comparison to make is between related sequences, such as point mutations, for which there are well-

documented differences in function. For example, a mutation in the Pribnow box may create a weaker promoter, or might change an operator sequence into a constitutive operator.

Fairly recently, the structure and properties of the *trp* operator were described (Lefèvre *et al.*, 1987, 1988), for which there are both ‘*in vitro*’ and ‘*in vivo*’ binding data for the interaction with the *trp* repressor. A structure of the *trp* operator–*trp* repressor complex has been determined by X-ray crystallography (Otwiniowski *et al.*, 1988), which shows that the structure and flexibility of the DNA is important for determining the specificity of the interaction. In the present paper the solution conformation of a mutant *trp* operator is examined, in which the mutation corresponds to a known operator constitutive mutation, and also a promoter down-mutation (Bennett & Yanofsky, 1978; Oppenheim & Yanofsky, 1980; Oppenheim *et al.*, 1980). ‘*In vitro*’ binding data show that the mutant operator binds the *trp* repressor with much lower affinity than the wild-type operator, though, because the affinity is still dependent on the presence of tryptophan, the binding is presumably specific (Chandler & Lane, 1988). Further, in the wild-type sequence there are temperature-dependent conformational transitions in the region of the Pribnow box, which occur at higher temperatures in the mutant (Lefèvre *et al.*, 1988; Lane, 1989). In the present paper, solution conformations of the mutant operator and a more detailed analysis of the conformational transitions are described, allowing a comparison between the two sequences to be made.

Abbreviations used: NOE, nuclear Overhauser enhancement; NOESY, two-dimensional nuclear-Overhauser-enhancement spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; rms, root mean square.

MATERIALS AND METHODS

Data collection

The 20-bp oligonucleotide dCGTACTGATT^{*}.AATCAGT-ACG was synthesized using the phosphoramidite method and purified as previously described (Lane, 1989).

N.m.r. spectra were recorded at 500 MHz on a Bruker AM 500 instrument equipped with an ASPECT 3000 computer, 16-bit digitizer and process controller. One-dimensional n.m.r. spectra were recorded over a 5 kHz spectral width using 16384 complex points and a 90° pulse width. Two-dimensional nuclear-Overhauser-enhancement spectroscopy (NOESY) spectra were recorded in the phase-sensitive mode using the time-proportional phase-incrementation scheme (Marion & Wüthrich, 1983) and a spectral width of 5000 Hz with 2048 points in F2 and 512 in F1, zero-filled to 2048 points before Fourier transformation. The free-induction decays were apodized in both dimensions using a 90°-shifted sine-squared bell function, which ensures that the free-induction decay smoothly approaches zero without affecting the intensity of the first point. Sine-modulated data were acquired, with appropriate calibration of the receiver phase, to ensure a flat base plane (C. J. Bauer & T. A. Frenkiel, unpublished work). NOESY spectra were recorded at different mixing times at three temperatures. For comparative purposes, spectra were obtained under identical conditions of acquisition at 10, 25 and 40 °C, using mixing times of 70, 100 and 150 ms respectively. These mixing times were inversely proportional to the tumbling time of the molecule at the three temperatures, as previously determined (Lane, 1989). Additional NOESY spectra were recorded at 40 °C with mixing times of 75 and 250 ms. Relative cross-peak volumes were determined from cross-sections, assuming that volumes are proportional to the product of the peak height and the line width in F2 and F1 (Chary *et al.*, 1988). For comparing NOESY intensities at different temperatures, the cross-peak volumes were normalized to those for the H-6–H-5 vectors of the cytosine residues, for which the cross-relaxation rate constants have been previously determined as a function of temperature by truncated one-dimensional NOE experiments (Lane, 1989). The series at 40 °C was also normalized to the volume of a single proton at a mixing time of zero, using selective T₁ data for resolved base protons as described by Mirau (1988).

Complete one-dimensional truncated NOE (Wagner & Wüthrich, 1979) data sets were obtained at different irradiation times at 25 °C (*t* = 50, 100, 200 300 and 500 ms) by irradiating each base proton (H-8/H-6), and each H-1' resonance in turn. The data sets for bases T⁹–T¹³ were checked by repeating the experiments on an independently synthesized sample of the oligonucleotide. The agreement of the NOE intensities between the two data sets was within ±10%. In addition, one-dimensional NOE data sets were acquired at 40 °C on irradiation of the H-8 and H-6 of the bases T⁹, A¹¹, A¹² and T¹³ to determine in greater detail the temperature-dependent conformational changes that occur in this section of the oligonucleotide.

J-resolved spectra were recorded at 40 °C with spectral widths of 4600 Hz in F2 and 153 Hz in F1; 8192 points were collected in F2, and 48 points in F1, zero-filled to 256 to yield final digital resolutions of 1.12 Hz per point in F2 and 0.56 Hz per point in F1. Free-induction decays were transformed using shifted sine-squared bell functions in both dimensions. The spectra were tilted and symmetrized for display. Splitting patterns for coupling of the H-1' to the H-2' and H-2'' protons were obtained from columns of the two-dimensional data set. HOHAHA experiments were run at high digital resolution (4.2 kHz spectral width, 4096 points in F2, 512 points in F1 with zero-filling to 8096 by 2048 points prior to Fourier transformation) at 500 MHz using a B1

field of 8 kHz for the spin lock produced with the MLEV-17 sequence (Bax & Davis, 1985; Flynn *et al.*, 1988).

Structure determination

The structural parameters describing the conformation of the oligonucleotides were determined using the program NUCFIT (Lane, 1990). The conformations of the individual nucleotides were initially determined from the NOE data, and the spin multiplicities and sums of coupling constants were obtained from the J-resolved and HOHAHA experiments.

To analyse the temperature-dependent conformational transitions that were previously observed in parts of the molecule (Lane, 1989), data were collected at three temperatures, and structures independently derived for each data set. For helical parameters 300–400 individual conformations were searched and optimized. The convergence of the fitting process was monitored with the function *R*, the mean absolute fractional deviation (Lane, 1990):

$$R = (1/N) \sum | [\text{NOE}(\text{obs.}) - \text{NOE}(\text{calc.})] / \text{NOE}(\text{obs.}) | \quad (1)$$

where *N* is the number of observations, NOE (obs.) is the observed NOE, and NOE(calc.) is the calculated NOE. All calculations were carried out on a Macintosh SE/30 computer.

RESULTS AND DISCUSSION

Nucleotide conformations

The resonances of all base and sugar protons (except H-5' and H-5'') were assigned previously (Lane, 1989). The principal parameters needed to describe the conformations of the nucleotide units are the glycosidic torsion angle (*χ*), the pseudorotation angle (*P*) and the fraction of the south state (*f_s*) (Rinkel *et al.*, 1987). A qualitative indication of the sugar pucker mode can be obtained from coupling patterns for H-1', which has two coupling partners, H-2' and H-2'', leading to characteristic splitting patterns for different puckers (Lefèvre *et al.*, 1987; Rinkel & Altona, 1987). The coupling patterns, and the sum of the coupling constants, $\Sigma_1 = {}^3J_{1,2'} + {}^3J_{1,2''}$, can be determined from rows parallel with F2 in high-digital-resolution HOHAHA spectra (Lane & Forster, 1989). Rows through the H-1'–H-2' cross peaks are shown in Fig. 1. Most of the splitting patterns show triplets, with the sum of the coupling constants, Σ_1 , larger than 14 Hz, which is typical of sugar conformations largely in the south domain (i.e. near C-2' endo) (Rinkel & Altona, 1987). However, pure C-2' endo produces double doublets, indicating either that the sugars are not pure states (e.g. a mixture of C-2' endo and C-3' endo) or are closer to C-1' exo or C3' exo. The splitting patterns are given in Table 1. The apparent coupling constants show that most of the pseudorotation phase angles lie in the south range (i.e. *P* = 120–198°). From the value of Σ_1 , a lower limit to the fraction of the south conformation can be estimated (Rinkel & Altona, 1987). As Table 1 shows, the purines tended to have a higher state of conformational purity than the pyrimidines, as previously noted for other sequences (Rinkel *et al.*, 1987; Bax & Lerner, 1988; Zhou *et al.*, 1988; Lane & Forster, 1989).

Of the proton–proton distances within the deoxyribose ring, only the 1'–4' and 2'–4' show a significant variation with the sugar pucker, and the latter distance is rather large for all pseudorotation phase angles greater than about 90° (van de Ven & Hilbers, 1988). The relative intensities of NOEs connecting the H-1' resonances to H-2'' and H-4' can be judged by taking rows parallel with F2 through H-1' as shown in Fig. 2. The resolved 1'–4' cross peaks are smaller than the resolved 1'–2'' cross peaks [where the distance is 0.23 nm (2.3 Å)]. For comparison, a row through the H-6 of bases C⁹ and C¹⁹, showing the cross-peaks to

Fig. 1. Multiplets showing spin-spin coupling between H-1' and H-2'

The HOHAHA spectrum was recorded at 313 K with 4096 points in F2 and 512 in F1 over a spectral width of 4200 Hz. The data matrix was zero-filled to 8192 points in F2 and 2048 in F1 prior to apodization with a sine-squared function shifted 60° in both dimensions and Fourier transformation. Cross sections were parallel with F2 through the H-1'-H-2' cross-peak.

the H-5 resonances, is shown [distance = 0.246 nm (2.46 Å)]. Extensive simulations of the dependence of the NOE intensities on the pseudorotation phase angle showed that these NOEs are not significantly contaminated by spin diffusion (which would tend to increase the NOE intensity), though naturally, because the relaxation of the H-2' is much more rapid than that of H-4', the ratio of the intensities will tend to underestimate the true distance H-1'-H-4'. However, comparison of the intensities with the intensity of the cytosine H-6-H-5 cross-peaks shows that the H-1'-H-4' distance must be larger than 0.3 nm (3 Å), which is inconsistent with phase angles in the range $60 < P < 120^\circ$ (van de Ven & Hilbers, 1988). Further, the H-2''-H-4' cross-peaks are of very low intensity (not shown), also indicating phase angles near C-2' endo, with a relatively small fraction of the C-3' endo state. Hence, an equilibrium mixture of C-2' endo and C-3' endo pucker states represents the simplest model consistent with all of the data.

More detailed analysis of the NOE intensities for H-1'-H-2'

Table 1. Spin multiplicities of H-1' resonances

Multiplicities were determined at 40 °C from HOHAHA spectra (cf. Fig. 1): T, triplet; D, doublet; DD, double doublet.

Base	δ (p.p.m.)	Multiplicity	Σ_1 (Hz)	f_s
C ¹	5.79	T	15.2	0.91
G ²	5.97	T	15.0	0.9
A ⁴	6.21	T	14.8	0.85
C ⁵	5.78	DD	15.6	1.0
G ⁷	5.50	DD	14.7	
			8.6, 6.1	1.0, $P_s = 195$
T ¹⁰	5.71	T	14.5	0.8
A ¹¹	5.93	T	13.8	0.67
T ¹³	5.83	T	15.0	0.9
C ¹⁴	5.55	T	14.3	0.75
A ¹⁵	5.96	DD	15.5	0.95
G ¹⁶	5.83	T	14.5	0.8
A ¹⁸	6.20	T	15.0	0.9
C ¹⁹	5.65	T	15.0	0.9
G ²⁰	6.17	T	15.0	0.9

showed that this vector shows internal motion requiring an order parameter in the range 0.7–0.8, similar to estimates made on other oligodeoxynucleotides (Lane & Forster, 1989).

The sugar conformations can be further analysed by using the NOE. One-dimensional NOE time courses were obtained by irradiating the H-8 and H-6 resonances of purine and pyrimidine residues respectively, giving NOEs to H-1', H-2', H-2'' and H-3'. Except for the NOE to H-2', these NOEs are contaminated by spin diffusion, even at short irradiation times. The data were therefore analysed by integrating the Bloch-Solomon equations for a system of spins corresponding to a complete nucleotide. The data were analysed in two ways. First, the sugar pucker was assumed to be unique, i.e. a single value of the pseudorotation phase angle, P , and the glycosidic torsion angle, χ , suffices to describe the conformation. Second, the sugars were assumed to exist as a mixture of C-2' endo ($P = 162^\circ$) and C-3' endo ($P = 18^\circ$), so that the parameters to be determined are χ and f_s , the fraction C-2' endo. In the majority of cases, best fits were obtained for a mixture of south and north states. In all nucleotides, the glycosidic torsion angles lie in the anti range (i.e. near -110°). Because the number of observations (≈ 20) greatly exceeds the number of parameters (2) for each nucleotide, the parameters describing the nucleotide conformations can be considered to be well determined. Fig. 3 shows observed and calculated time courses for two nucleotides, showing the quality of the fits. The nucleotide conformations at 298 K are given in Table 2.

As the data in Table 2 show, all nucleotides are in the anti range of glycosidic torsion angles, with a mean value of $-112.5 \pm 5.5^\circ$. There is a slight tendency of the purine nucleotides to adopt a larger angle ($-106 \pm 6.7^\circ$) than the pyrimidines ($-115 \pm 5.7^\circ$), but the difference is only marginally significant statistically. The NOE data are also consistent with all nucleotides being predominantly near C-2' endo, with the pyrimidines showing a greater tendency to adopt the C-3' endo conformation for a significant fraction of the time. The fraction C-2' endo is relatively poorly determined, however (probable error ± 0.2), and agrees only qualitatively with the coupling-constant data. Nevertheless, the mean fractional error, R , for all 20 nucleotides is 0.11, which is within the experimental precision. Hence, the conformations that have been determined account for all of the measured intranucleotide NOEs.

Fig. 3. Determination of nucleotide conformations

Nucleotide conformations were found by non-linear regression and conformational space searching as described in the text. (a) Best conformation for T^{13} . NOEs were observed at H-1', H-2', H-2'' and H-3' on saturation of H-6. Lines are regression fits with the parameters given in Table 2. (b) Best conformation of A^4 . NOEs were observed at H-1', H-2', H-2'' and H-3' on saturation of H-8. Lines are regression fits with the parameters given in Table 2.

Internucleotide conformations

The relative conformation of two base-pairs needs several parameters for a unique description: the rise, twist, interbase pair roll, propellor twist, tilt, shift and slide, i.e. a total of eight parameters (there is a propellor twist for each base-pair), therefore requiring at least eight independent experimental observations. For each dinucleotide, it is possible to measure NOE time courses for the H-1', H-2', H-2'', and H-3' of base i to the H-8/H-6 of base $i+1$ and, for neighbouring imino protons, giving up to nine NOE time courses for each pair of dinucleotides. NOEs between the imino protons of neighbouring AT base-pairs were not used, owing to the spectral overlap. In addition, in AA steps, an NOE can be measured between the H-2 protons, in NC steps, an NOE between the H-8/H-6 and H-5 and in NT steps between H-8/H-6 and TMe. Pairs of dinucleotides were constructed in which the individual nucleotides were initially constrained in the conformations found above. Because the 20 bp fragment is

Fig. 2. Intra-ring D-ribose NOEs from NOESY spectroscopy

The phase-sensitive NOESY spectrum was recorded at a mixing time of 100 ms as described in the Materials and methods section. The data matrix (2048×512 points) was zero-filled to 4096 by 2048 points before apodization by a 90° -shifted sine-squared function in both dimensions. The spectral width was 5000 Hz, and the temperature was 298 K. The cross-sections are taken through the diagonal peaks (H-1') parallel with F2, showing NOE intensities between: (a) H-1' and H-4', H-2'' and H-2'; (b) H-6 and H-5 of C^5 and C^{19} .

Table 2. Nucleotide conformations at 298 K

χ and f_s were determined from intranucleotide NOE time courses as in Fig. 3, using the program NUCFIT. P_s was set to 162° for purines, except G⁷, where it was 195°, and 144° for pyrimidines. P_N was set to 18° and the pseudorotation phase angle to 37°. χ is the glycosidic torsion angle, f_s is the mole fraction of the south state ($P_s = 144$ –196°) and R is mean absolute fractional error. The statistical error on χ is $\pm 3^\circ$, except where stated otherwise. The error in f_s is about ± 0.2 . (* indicates an error of $\approx \pm 5^\circ$).

Base	$-\chi$	f_s	R
C ¹	116	0.7	0.096
G ²	113	0.83	0.144
T ^{3*}	120	0.89	0.094
A ⁴	102	0.95	0.075
C ⁵	118	0.71	0.164
T ⁶	112	1.0	0.07
G ⁷	113	1.0	0.13
A ⁸	107	1.0	0.115
T ⁹	113	0.72	0.043
T ^{10*}	120	0.6	0.167
A ¹¹	100	1.0	0.07
A ¹²	115	0.9	0.08
T ¹³	116	1.0	0.095
C ¹⁴	120	0.8	0.13
A ¹⁵	111	1.0	0.133
G ^{16*}	107	0.6	0.146
T ¹⁷	108	0.6	0.108
A ¹⁸	106	1.0	0.087
C ¹⁹	120	0.79	0.144
G ²⁰	111	0.7	0.141

significantly anisotropic, the cross-relaxation rate constants depend on the angle the interproton vector makes with the long axis of the molecule. Assuming an average rise of 0.34 nm (3.4 Å) per base-pair, the ratio of the two correlation times is about 2.8. The appropriate correlation times are calculated from those measured for the cytosine H-6–H-5 vectors, as previously described (Lane, 1990).

Initially, NOE time courses were generated for numerous sets of parameters (typically 200–300 sets), with least-squares optimiz-

Fig. 4. Determination of helical parameters

NOE intensities were obtained at 298 K on irradiation of T⁹ H-6 and T¹³ H-6, which were simultaneously fitted with NUCFIT as described in the text. The nucleotide conformations were as given in Table 2. The curves were obtained for rise = 0.36 nm (3.6 Å), twist = 34.5°, tilt = 5°, roll = 10°, slide = 0.05 nm (0.5 Å) and shift = 0. The R factor was 0.07. ■, NOEs observed at H-1', H-2', H-2'' and H-3' of A⁸ on saturation of T⁹ H-6; —, fitted curve; □, NOEs observed at H-1', H-2' and H-2'' of A¹² on saturation of T¹³ H-6; ----, fitted curve.

ation of the helical twist and reciprocal T_1 values to minimize the deviation between observed and calculated NOEs. Upper limits to separation were imposed based on the maximum distance between the C-3'(i) and the C-5'(i+1) [0.52 nm (5.2 Å)]. Lower limits were set by van der Waals distances. Where available, imino-proton NOEs were used to place upper limits on the rise. Parameter sets that gave the smallest value of the mean absolute deviation (R), no van der Waals violations, distance from C-3'(i) to C-5'(i+1) less than 0.52 nm (5.2 Å) and good visual agreement of the calculated NOE intensities with the observed intensities, were used for further refinement. The search in parameter space also serves to define the co-variance among the parameters. The conformations were then refined as base-pairs in which the glycosidic torsion angles of the 5' base, the rise, twist, tilt and shift were allowed to vary freely. Fig. 4 shows selected time courses and fits for a pair of dinucleotides in which 33 NOE intensities were simultaneously fitted.

It was found that, in general, several sets of parameters accounted for the data, so that only ranges for parameter estimates can be given, as opposed to a single set of parameters with standard deviations. The parameters are given in Table 3. Although the helical twist is fairly well-determined in the statistical sense (fitting error typically around $\pm 3^\circ$), the helical rise is often relatively poorly determined, mainly because of covariance with other parameters. Optimum fits are usually found for values of the rise in a 0.05 nm (0.5 Å) interval. Similarly, tilt angles are quite well determined, typically within about 5°, but the base-pair roll is generally underdetermined, with optimum fits found within an interval of around 20°. The exception is the A¹¹–A¹² base-pair roll, which is fixed largely by the NOEs between the C-2 protons in the minor groove. Simulations have shown that these NOEs are very sensitive to the base-pair roll, but comparatively insensitive to the rise, twist and tilt. The average helical twist is $35 \pm 3.5^\circ$ for all base-pair steps (i.e. 10.3 bp/turn), or $35.5 \pm 3^\circ$ if the first step is ignored. Despite the low precision of many of the internucleotide parameters, the molecule clearly belongs to the B-family of conformations, and the variation about the canonical B-structure is relatively small. Fig. 5 shows the variation of the helical twists along the sequence. The helical twists predicted by the procedure of Tung & Harvey (1986), and those determined by Otwinowski *et al.* (1988) for a complex of the *trp* holorepressor with a symmetrized operator are included for comparison. The largest deviations among the

Table 3. Inter-nucleotide parameters

Inter-nucleotide parameters were determined by extensive conformation-space searching and optimization as described in the text. Because of insufficient independent data, the base-pair shift was held constant at 0°. The values of the tilt, slide and roll are given as ranges, in which a large number of conformations were consistent with the data. The final value of R for the entire molecule was 0.108. (Note 1 Å = 0.1 nm.)

Step	Rise (Å)	Twist (°)	Tilt (°)	Slide (Å)	Roll (°)	R
C ¹ –G ²	3.7 ± 0.3	29 ± 5	–10	0/–1	0/–20	0.093
G ² –T ³	3.5 ± 0.3	38 ± 3	0/–10	0/1	0/–20	0.09
T ³ –A ⁴	3.6 ± 0.4	35 ± 5	–5/–10	–1/1	0/–20	0.14
A ⁴ –C ⁵	3.4 ± 0.4	33 ± 3	0/–10	–1/1	0/–20	0.11
C ⁵ –T ⁶	3.4 ± 0.2	31 ± 3	–5/–10	–0.5/1	0/–20	0.09
T ⁶ –G ⁷	3.4 ± 0.3	34 ± 4	–5/–10	0/–1	0/–20	0.12
G ⁷ –A ⁸	3.4 ± 0.3	40 ± 4	0/–5	–1/1	0/–20	0.11
A ⁸ –T ⁹	3.6 ± 0.3	35 ± 2	–5/–10	–0.5/0.5	0/–20	0.06
T ⁹ –T ¹⁰	3.4 ± 0.4	34 ± 4	0/–10	0/–0.5	–5/–20	0.12
T ¹⁰ –A ¹¹	3.7 ± 0.3	40 ± 3	0	0	–10/–30	0.11

Fig. 5. Comparison of experimental and predicted helical-twist angles

■, Helical twists determined by n.m.r. (Table 3); ----, helical twists predicted by the method of Tung & Harvey (1986); □, helical twists observed in the co-crystal of a *trp* operator with the *trp* holorepressor.

data sets occur at the steps $T^3 \cdot A^{18}/A^4 \cdot T^{17}$ and $T^{10} \cdot A^{11}/A^{11} \cdot T^{10}$. The exact cause of these differences is not clear, but it should be noted that the central AT steps are those involved in temperature-dependent conformational transitions (i.e. are relatively flexible, see below), and the absolute precision of the n.m.r. method is unlikely to be better than $\pm 5^\circ$. The helical twists predicted by the method of Tung & Harvey (1986) or by the Calladine–Dickerson rules (Calladine, 1982; Dickerson, 1983) gave rms agreement with the experimental data of 6.3° and 7.4° respectively, while the rms agreement with the *trp* holorepressor–operator complex is 4.7° (omitting the first step which is different), suggesting that there is only minor distortion of the DNA in the complex with the repressor.

Temperature-induced conformation changes

The molecule has previously been shown to exist in at least two conformational states whose populations are strongly dependent

on temperature. Several n.m.r. parameters showed that the conformational transitions occurring at 16°C and near 40°C are localized to about four bases near the centre of the molecule (TAAT). To examine in greater detail the nature of the conformational transitions in these conformational states, NOESY spectra were recorded at 10, 25 and 40°C at mixing times, t_m , inversely proportional to the independently determined overall correlation time (Lane, 1989). In a two-spin system, A and B, the cross-peak intensity $a_x(t_m)$ is given by:

$$a_x(t_m) = [M^0 \sigma / (\lambda_1 - \lambda_2)] \{ \exp(\lambda_1 t_m) - \exp(\lambda_2 t_m) \} \quad (2)$$

(where M^0 is equilibrium magnetization) where:

$$2\lambda_{1,2} = -(\rho_A + \rho_B) \pm [(\rho_A + \rho_B)^2 - 4(\rho_A \rho_B - \sigma^2)]^{1/2} \quad (3)$$

and ρ_A and ρ_B are the spin-lattice relaxation rate constants and σ is the cross-relaxation rate constant. For slowly tumbling molecules, the relaxation rate constants ρ_A , ρ_B and σ are dominated by the $J(\Delta\omega)$ terms, which for protons is essentially equal to the correlation time. Provided that external (leakage) relaxation is small compared with dipole–dipole relaxation, which is generally true for large molecules (Neuhaus & Williamson, 1989), then all rate constants in eqns. (2) and (3) are directly proportional to the correlation time. Hence, in a rigid body in which the conformation is independent of temperature, an increase in temperature that causes a 2-fold reduction in correlation time will require a 2-fold increase in the mixing time to retain the same NOE intensities. This is also true for large numbers of spins, when all rates are directly proportional to the correlation time, as has been verified by extensive stimulation for spin systems found in nucleic acids (Bauer *et al.*, 1990). If, however, changing the temperature also changes the conformation, then the r^{-6} terms in the relaxation rate constants are not constant, and the observed cross-peak intensity will not be independent of the temperature when recorded at the appropriate mixing time.

Table 4 compares the normalized cross-peak intensities at

Table 4. Relative NOE intensities at three temperatures

NOESY cross-peak intensities were normalized to the averaged volume of the four cytosine H-6–H-5 cross-peak volumes as described in the text. The values correspond to temperatures of 10, 25 and 40°C at mixing times of 70, 100 and 150 ms respectively. –, Not determined. Error on volume ratios is approx. $\pm 40\%$ for H-1', 20% for H-2' and 30% for H-2'' and H-3'. Intranucleotide intensities: * denotes that relative intensities change significantly between 25 and 40°C ; † denotes significant change between 10 and 25°C .

Base	NOE for H-8/H-6 to:			
	H-1'	H-2'	H-2''	H-3'
C ¹	0.35/0.3/0.3	1.4/1.3/1.0	–/0.3/0.4	–/0.2/–
G ²	0.15/0.14/0.1	0.35/0.4/0.55	0.2/0.3/0.2	–/–/0.1
T ^{3*}	0.1/0.15/0.2	0.6/0.75/1.3	–/0.55/–	–/–/–
A ⁴	0.4/0.2/0.2	0.8/0.7/0.9	0.4/0.3/0.55	0.2/0.15/0.25
C ⁵	0.5/0.4/0.4	1.3/1.0/1.4	0.7/–/0.3	–/–/–
T ⁶	0.1/0.1/0.25	–/1.2/1.4	–/0.45/0.45	–/–/0.3
G ⁷	–/0.1/0.15	–/0.45/0.4	–/0.25/0.2	–/–/–
A ^{8†}	0.1/–/0.2	0.6/1.1/1.0	–/0.3/0.4	–/0.2/0.15
T ⁹	–/0.15/0.35	–/0.6/0.9	–/0.3/0.2	–/–/0.25
T ^{10*}	0.1/0.2/0.2	0.5/0.6/1.3	–/0/–	–/0.15/–
A ^{11*}	0.2/0.2/0.3	–/1.0/1.4	0.6/0.55/0.8	–/0.2/–
A ^{12*}	0.1/0.1/0.2	0.5/0.5/0.8	–/–/0.3	0.1/0.1/0.05
T ^{13*†}	0.1/0.1/0.25	0.3/0.6/0.8	0.35/0.25/0.3	–/–/–
C ¹⁴	–/–/–	1.6/1.5/1.5	–/1.0/–	–/–/–
A ¹⁵	0.2/0.2/0.25	–/–/0.9	0.6/0.4/0.4	–/0.2/0.15
G ¹⁶	–/0.1/0.1	–/0.4/0.25	–/0.2/0.1	–/0.1/0.05
T ¹⁷	0.1/0.1/0.2	0.89/1.0/1.2	–/0.45/0.4	–/–/0.3
A ^{18*†}	0.2/0.1/0.25	0.8/0.7/1.3	0.45/0.3/0.6	0.25/0.15/0.15
C ¹⁹	0.25/0.2/0.5	1.25/0.95/1.4	0.7/–/0.3	–/–/–
G ²⁰	0.1/0.05/0.1	0.6/0.55/0.5	–/0.15/0.15	–/–/–

Fig. 6. Differences in glycosidic torsion angles between 25 and 40 °C

Data were analysed as described in the text. The glycosidic torsion angles for the data set at 298 K were taken from Table 2, and the apparent glycosidic torsion angles at 40 °C were determined from NOESY spectra as described in the text. The difference is plotted as $\chi(313) - \chi(298)$. The experimental precision for the data set is $\pm 3^\circ$ at 298 K and $\pm 5^\circ$ at 313 K.

three temperatures. Because of the relatively low resolution of the spectra at 10 °C, only a qualitative discussion of the conformational changes that occur between 10 and 25 °C is possible. Significant changes in relative NOESY intensities are observed in every residue from A⁸ to T¹³, and also in the base-pair T⁹-A¹⁸. The largest changes occur between 25 and 40 °C in the central T⁹-A¹² region. A quantitative analysis of the temperature-dependence of the conformation was made at five points in the molecule, using detailed one-dimensional NOE time courses for the cytosine residues C⁵ and C¹⁹, and the residues T⁹, A¹¹, A¹² and T¹³. In addition, NOESY spectra were recorded at 40 °C with mixing times of 75, 150 and 250 ms. The NOESY data at 40 °C were analysed to give the apparent glycosidic torsion angles, from which the changes from 25 °C could be calculated. The changes are shown in Fig. 6. Given that differences of 5° or less are not statistically significant, the largest changes are concentrated in the central region of the molecule from T⁹ to C¹⁴. Similarly, inter-nucleotide NOE intensities are significantly different only for base-pairs in this region, in agreement with previous findings for other n.m.r. parameters (Lane, 1989). It seems likely, then, that substantial conformational changes occur mainly in the Pribnow box part of the sequence (i.e. T⁹-T¹³). For this reason, complete one-dimensional data sets were acquired at 40 °C only for these nucleotides, plus C⁵ to act as a reference, from which the nucleotide conformations and helical parameters could be determined. The analysis of the intranucleotide conformations is given in Table 5, where they are compared with the results for the data obtained at 298 K. Clearly, these results confirm the findings of the less precise NOESY data, that there are indeed significant conformational transitions in the Pribnow-Box region of the molecule.

However, although the molecule appears to be essentially in a single conformation at 25 °C, this not true for A¹¹ and A¹² at 40 °C, as the line width of A¹¹ H-2 is at a maximum at 40 °C, consistent with this nucleotide being near the midpoint of a second transition (Lane, 1989). Analysing the NOE data as a single conformation must produce virtual conformations (Jardetzky, 1980). Unfortunately it is not possible to acquire data at sufficiently high temperature to force the molecule into a single, high-temperature, conformation, because of exchange of base protons for deuterons, and possible strand separation. Nevertheless, the direction of changes of the apparent parameter values (which are underestimates of the true magnitudes of the changes) will be indicators of the conformational differences that

Table 5. Change in glycosidic torsion angle from 298 to 313 K

Glycosidic torsion angles were determined from one-dimensional NOE time courses as described in the text.

Base	$\chi(298) (^\circ)$	$\chi(313) (^\circ)$	$\chi(313) - \chi(298) (^\circ)$
C ⁵	-118	-119	-1
T ⁹	-113	-105	8
A ¹¹	-100	-90	10
A ¹²	-115	-102	13
T ¹³	-114	-108	6

occur between the high- and low-temperature conformations. Additional calculations (not given) based on the assumption that at 40 °C the main conformational transition has an equilibrium constant of 1 indicate that the apparent changes given in Fig. 6 underestimate the true changes by about a factor of 2.

A similar analysis was made of the inter-nucleotide NOE intensities. Although the results will clearly underestimate the changes from the conformation present at 298 K, they should give a reliable indication of the direction of the changes. The helical parameters at 40 °C are given in Table 6, along with the changes from the 25 °C structure.

It can be seen that the nucleotides whose conformations change the most are those of A¹¹ and A¹². Because of the low precision of the estimates of the slide and roll, it is difficult to distinguish conformational differences even at the level of 0.1 nm (1 Å) for the slide or 15° for the base-pair roll. However, within the experimental limits, it appears that the orientations at the steps A⁸-T¹³/T⁹-A¹² and T⁹-A¹²/T¹⁰-A¹¹ do not change significantly, whereas the step T⁹-A¹²/T¹⁰-A¹¹ does. There is substantial decrease in the helical twist at this step, and also a decrease in the base-pair roll. Although the base-pair roll is not well determined in general, in AA steps an extra NOE is readily observed (adenosine H-2 to H-2), which is very sensitive to the roll angle and rather insensitive to the helical twist, rise and tilt. The data in Table 6 suggest a flattening of the roll angle of about 10° as the temperature is raised, which would lead to an increase in the H-2-H-2 distance of about 0.04 nm (0.4 Å). The change in the distance measured previously was about 0.03 nm (0.3 Å) (Lane, 1989). As we have previously argued, these highly localized conformational changes lead to changes in base stacking, which presumably accounts for the strong temperature-dependence of the conformational transitions (Lefèvre *et al.*, 1988).

Comparison with the wild-type operator

Chemical shifts and relaxation rate constants give qualitative information about conformation. Fig. 7 shows the changes in the chemical shifts of protons in the unsubstituted nucleotides of the mutant versus the wild-type sequence. Differences of chemical

Table 6. Inter-base-pair parameters at 313 K

Parameters were determined from one-dimensional NOE time courses as described in the text (note 1 Å = 0.1 nm).

Step	Rise (Å)	Twist (°)	Twist(313)- twist(298) (°)	Tilt (°)	Slide (Å)	Roll (°)	R
A ⁸ -T ⁹	3.6 ± 0.3	35 ± 2	0	-5/-10	-0.5/0.5	0/-15	0.07
T ⁹ -T ¹⁰	3.3 ± 0.3	23 ± 3	-11	-5/-10	-0.5/-1	-10/10	0.05
T ¹⁰ -A ¹¹	3.8 ± 0.3	40 ± 3	0	0	-0.5/0.5	-15/-30	0.08

Fig. 7. Changes in chemical shift between the symmetrized wild-type and mutant *trp* operators

Chemical shifts for the wild-type operator were taken from Lefèvre *et al.* (1987) and for the mutant from Lane (1989). The chemical-shift differences are mutant minus wild-type. Chemical shifts are accurate to ± 0.01 p.p.m. for the base protons, and ± 0.02 p.p.m. for the sugar protons. (a) Base protons (H-8, H-6); (b) sugar protons: ■, H-1'; □, H-2'; ▨, H-2''.

shift of less than ± 0.05 p.p.m. are insignificant. Changes in chemical shift were found to be insignificant at more than two bases removed from the mutation site, and in general, the changes are larger for protons in the minor groove than those in the major groove. Similarly, selective T_1 measurements at 40 °C on resolved base protons are very similar in the two sequences (results not shown). The conformations of the two molecules appear to be very similar, at least at two or more nucleotides removed from the sites of mutation.

The conformation of the symmetrized wild-type *trp* operator was analysed previously (Lefèvre *et al.*, 1987, 1988) using a different means of data analysis in which the puckers of the pyrimidine residues were intermediate between C-3' endo and O-4' endo. The purines, however, were largely in the south domain, and showed a high degree of conformational purity ($f \geq 0.9$). The data for the purines, which are more extensive than for the pyrimidines (the spectral resolution of the pyrimidines was relatively poor in the wild-type sequence), have been re-analysed with NUCFIT, assuming a mixture of puckers, anisotropic rotation and order parameters for the H-1-H-2'' vectors of 0.75. In addition, sufficient data were also obtained for the nucleotides T⁹ and T¹⁰. The results are given in Table 7. For the purines, the agreement between the two analyses was in most cases within 5° for the glycosidic torsion angle. However, significant differences were found for T⁹ and T¹⁰. The reason for this appears to be that the previous analysis placed the puckers of these nucleotides in the north domain, whereas the current analysis the pucker is found primarily in the south domain with a small admixture of the north conformer. This difference is sufficient to account for the change in Table 7.

Table 7. Comparison of the wild-type operator with the mutant operator at 298 K

Glycosidic torsion angles for the mutant (Oc) sequence are taken from Table 2, whereas those for the wild-type sequence (O) were re-analysed using the data given in Lefèvre *et al.* (1987) as described in the text.

Base	$\chi(O)$	$\chi(Oc)$	$\chi(Oc) - \chi(O)$
G ²	-102	-113	-11
A ⁴	-103	-102	1
G ^{7*}	-113	-113	0
A ^{8†}	-110	-107	3
T ⁹	-116	-113	3
T ¹⁰	-116	-120	-4
A ¹¹	-100	-100	4
A ¹²	-102	-115	-13
A ¹⁵	-109	-111	-2
G ¹⁶	-108	-107	-1
A ¹⁸	-102	-106	-4
G ²⁰	-107	-111	-4

* A⁷ in Oc.

† G⁸ in Oc.

As Table 7 shows, the glycosidic torsion angles are very similar in both molecules, with two exceptions, namely G² and A¹². In the wild-type sequence, the second conformational transition has an apparent midpoint temperature of about 30 °C (Lefèvre *et al.*, 1988), whereas in the mutant sequence it is near 40 °C. This would account for the difference between the conformations of A¹². The reason for the discrepancy for G² is not clear, but may be a consequence of the relatively low precision of conformational analysis for this residue. Nevertheless, the differences are (except for A¹²) all less than 10°, which is about the limit of the experimental precision. Particularly interesting is that the conformations of the mutated nucleotides A⁷G and G⁸A are unaltered. It is likely that in linear, unconstrained, DNA fragments, the effects of base substitution have at most local effects on conformation, limited largely to the nearest neighbouring bases. Base substitutions are readily accommodated by relatively minor adjustments in the nearest neighbours, and these changes are not propagated far along the helix. This in fact justifies models of linear DNA conformation that assume simple nearest-neighbour effects (Calladine, 1982; Dickerson, 1983; Tung & Harvey, 1986).

There are several factors to take into consideration when investigating the effects of mutations on affinity for a specific protein. One is that particular functional groups must be present to make contact (either directly or indirectly via water-mediated contacts) with the complementary group in the protein. For example, exchange of an A for a G will replace an amino group (hydrogen-bond donor) by a carbonyl group (hydrogen-bond acceptor) in the major groove. This may occur with minimal influence on the conformation (see above). A second possibility is that the functional group may be present, but not in exactly the correct position or orientation. For example, replacing G by T may affect the local conformation while retaining the carbonyl group in the major groove. A third factor is flexibility, in which exchange of one base-pair for another may make the DNA locally less deformable by the protein, thereby costing extra energy (Otwinowski *et al.*, 1988). Although it is not possible to control conformation and functional-group chemistry completely independently, the present results, and the proposals of Calladine (1982), Dickerson, (1983) and Tung & Harvey (1986) suggest

that it may be possible to make a partial separation of these effects by analysis of appropriate mutations.

The mutation studied here probably falls into the first class for the following reasons. The four bases that appear to affect operator function most strongly are C⁵, T⁶, A⁷ and G⁸ (Bass *et al.*, 1988). Although there is some disagreement as to the exact position of the operator site recognized by the *trp* repressor (Staacke *et al.*, 1990), the sequence studied here encompasses the most mutationally sensitive bases, which occur in both proposed recognition sites (Bass *et al.*, 1988; Staacke *et al.*, 1990); the following arguments are independent of the exact position of the operator site. The *trp* repressor has similar affinity for each of the three natural operators (Klig *et al.*, 1988), where A⁷ is exchanged for C and G⁸ is exchanged for T. Conversely, the substitution A⁷G, G⁸A causes a much larger decrease in affinity (Chandler & Lane, 1988). Indeed, according to Bass *et al.* (1988), exchange of A⁷ for either G or T results in complete loss of operator function, whereas the substitution A⁷C causes only a slight loss of operator function. Given that the conformation around this site is not greatly affected by the mutation, it is likely that the amino group present in both A and C is required, and cannot be replaced by the carbonyl in G and T. Similarly, the 8-position requires a carbonyl (G or T), but cannot tolerate an amino group. Although it cannot be argued that the interactions between the protein and the DNA involve hydrogen-bonding alone, it seems likely that the interaction is governed largely by chemical determinants at positions 7 and 8.

In contrast, position 6 (T) will accept C, but neither purine. This would appear to be an example of the second class, where the precise conformation is more important than the local chemistry. On the other hand, position 5 (C) appear to require a C absolutely, which may indicate the requirement for both the correct conformation and a specific functional group. It will be of considerable interest, therefore, to examine the conformations of mutants T⁶C (similar conformation, different chemistry) and T⁶A (different conformation, similar chemistry).

Conclusions

The conformation of the 20-bp mutant *trp* operator is clearly B-like, showing relatively small deviations from the canonical B-structure. The nucleotide conformations are well defined, owing to the high ratio of data to parameters. In contrast, the inter-nucleotide parameters are generally much less well determined. The average helical twist is equivalent to 10.3 bp/turn. The overall mean fractional error, *R*, was 0.108 for a total of 560 NOE intensities, indicating that the range of conformations found adequately accounts for the experimental data.

In the conformation transition occurring near 40 °C, substantial changes in the conformations of A¹¹ and A¹² occur, which appear to be compensated for by an unwinding and a decrease in the roll angle at the T⁹-A¹²:T¹⁰-A¹¹ step. The conformation of the mutant seems to be quite similar to that of the wild-type, which is expected for exchange of a purine for a purine (Calladine, 1982; Dickerson, 1983; Tung & Harvey, 1986), though the mutant binds the *trp* repressor with greatly reduced affinity (Chandler & Lane, 1988), which can be traced to the swapping of the amino and oxo groups in the major groove. The main differences appear to be in the conformational transitions in the central region, and the flexibility of this region.

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