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Interaction of the *trp* repressor with *trp* operator DNA fragments

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Abstract. The interaction of the *trp* repressor with several *trp* operator DNA fragments has been examined by DNA gel retardation assays and by circular dichroism, in the absence and presence of the corepressor L-tryptophan. The holorepressor binds stoichiometrically to both the *trpO* and *aroH* operators, forming 1:1 complexes. In the presence of excess protein, additional complexes are formed with these operator fragments. The relative electrophoretic mobilities of the 1:1 complexes differ significantly for *trp* and *aroH* operators, indicating that they differ substantially in gross structure. A mutant trp operator, *trpO*^{c}, has low affinity for the holorepressor, and forms only complexes with stoichiometries of 2:1 (repressor: DNA) or higher, which have a very low electrophoretic mobility. Specific binding is also accompanied by a large increase in the intensity of the near ultraviolet circular dichroism, with only a small blue shift, which is consistent with significant changes in the conformation of the DNA. Large changes in the chemical shifts of three resonances in the ^{31}P NMR spectrum of both the *trp* operator and the *aroH* operator occur on adding repressor only in the presence of L-tryptophan, consistent with localised changes in the backbone conformation of the DNA.

Key words: *trp* repressor – *trp* operator – Circular dichroism – Gel retardation

Introduction

The trp repressor of *Escherichia coli* is a dimeric protein consisting of two identical subunits of molecular weight 12 500. The aporepressor has low, non-specific affinity for DNA, but in the presence of the corepressor L-tryptophan, the holorepressor recognises and binds to three operator sequences, *trpO*^{1}, *trpR* and *aroH*, which show high sequence homology over a twenty base-pair region. These sequences, and the corresponding *trp* operator from other enteric bacteria, allow a consensus operator to be defined (Gunsalus and Yanofsky 1980, Oppenheim et al. 1980). Direct binding studies using the DNA gel-retardation assay and DNAse I footprinting show that a specific 25 base-pair stretch of DNA is protected, and that the dissociation constant of the trp holorepressor: operator complex is about 0.5 nM at low ionic strength (< 50 mM) (Carey 1988). More recently, Marmorstein et al. (1991) have developed an assay based on protection against alkaline phosphatase. They found that with 20-base-pair DNA fragments, the K_d for specific binding was approximately 5 nM, whereas the K_d for non-specific binding was about 10 μM. Using a filter-binding assay as well as the band-shift and footprinting assay, Klig et al. (1988) have shown that the affinity of the *trp* holorepressor is highest for the *trp* operator (K_d = 1 to 5 nM), and only about three times lower for the *trpR* and *aroH* operators. Further, the *Rsa I* restriction protection assay gives a K_d for the trp holorepressor-operator complex of around 2 to 5 nM (Joachimiak et al. 1983).

However, based on an in vivo binding assay, Gunsalus and coworkers have suggested that for *aroH* and *trp* there may be tandem binding of repressor dimers to neighbouring sites (Kumamoto et al. 1987), similar to the interaction of *metJ* with DNA (Phillips et al. 1989, Phillips 1991). Recently, Müller-Hill and associates (Staacke et al. 1990) have taken up the idea of tandem repressor sites, and have suggested that the true *trp* operator site is shifted 4 base-pairs from that suggested by the sequence homology, and implicated by the DNAse I footprinting. Both sites, however, contain the tetrameric subsequence CTAG which has been shown to be the most sensitive to mutations in terms of operator function (Bass et al. 1987, 1988), and which is also implicated as being important in the co-crystal structure of the holorepressor with a synthetic operator (Otwinowski et al. 1988). Recent work suggests that this sequence in fact binds two molecules of the trp repressor dimer, whereas the canon-

Abbreviations: CD circular dichroism, *trpO*, *trpR*, *aroH* trp operator fragments, *trpO*^{c}, *trp*MH mutant trp operator fragments.

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ical trp operator sequence binds only one dimer (Carey et al. 1991, Haran et al. 1992).

Many of the direct binding studies to date have been done mainly at extremes of salt concentration. The gel retardation assays have been performed at low ionic strength (ca. 20 mM), and also near to the isoelectric point of the protein (pI = 6), where its solubility is very low. These conditions are also likely to promote non-specific binding, as was observed by Carey (1988). The filter-binding assays were performed at high concentrations of salt (ca. 1 M), which may also have the effect of "precipitating" the protein onto the filter (Klig et al. 1987). We have undertaken an investigation of the interaction of the *trp* repressor with synthetic DNA chosen to mimic the operator sites based on sequence homology and that proposed by Staacke et al. (1990). The oligomers are too short to allow tandem binding. We have measured the stoichiometry and relative affinity of the repressor for these sequences under different conditions, particularly those of salt concentration. We have made use of the gel retardation assay, which is performed at pH values from 6 to 8, CD, in which the concentration of salt can be varied as desired, and ^{31}P NMR to monitor the formation of specific and non-specific complexes of the trp repressor with DNA. The gel retardation assays allow approximate affinities to be determined, as well as binding stoichiometries. Further, the mobility of the complexes gives information about the size of the complexes. CD is useful for determining dissociation constants and also provides some information about conformational changes in the oligonucleotide induced by binding protein ^{31}P NMR reports exclusively on the phosphate backbone of the DNA, which presumably interacts directly with side-chains of the repressor.

Materials and methods

Trp aporepressor was purified from an overproducing mutant as previously described (Joachimiak et al. 1983, Borden et al. 1991). The protein was >95% pure judging from SDS electrophoresis. Protein concentrations were determined optically using the relation 1 absorbance unit at 279 nm = 1.2 mg ml^{-1} (Joachimiak et al. 1983). Repressor activity was monitored using the Rsa I restriction-protection assay (Joachimiak et al. 1983).

Oligonucleotides were synthesised on an Applied Biosystems synthesiser, and purified by FPLC. The oligonucleotides were annealed by heating to 90 °C for 5 minutes in a buffer containing 10 mM Na phosphate, 100 mM KCl, pH 7.0, and allowed to cool slowly. Duplex formation was checked by native acrylamide electrophoresis and ^{1}H NMR. The sequences used were:

trpO: d(CGTACTAGTTAACTAGTACG)$_2$
trpO^c: d(CGTACTGATTAATCAGTACG)$_2$
trpR: d(CGTACTCTTTAGCGAGTACA)·
d(TGTACTCGCTAAAGAGTACG)
aroH: d(ATGTACTAGAGAACTAGTGCAT)·
d(ATGCACTAGTTCTCATGTACAT)
*trp*MH: d(TAGCGTACTAGTACGCTA)$_2$

The oligonucleotides were dialysed against 10 mM Na phosphate, 100 mM KCl pH 7.5, and stored at −20 °C. Under these storage conditions, at high concentration (ca. 0.5 to 1 mM), the quantity of hairpin structures formed is insignificant. However, substantial quantities of hairpin forms were present on prolonged storage at 4 °C at low concentration and low salt concentrations. When desired, the hairpin form of *trpO* was prepared by heating 50 μM of the *trpO* DNA in 10 mM Na phosphate buffer pH 7.5 to 80 °C, and quenching on ice-water. Gel-electrophoresis of the product showed that essentially all of the DNA was in the hairpin form after this treatment. DNA concentrations were measured spectrophotometrically using extinction coefficients for the constituent oligonucleotides as described by Chandler and Lane (1988).

DNA gel-retardation assays were performed on 18 to 20% mini acrylamide gels in Tris-borate-EDTA (TBE) or Tris-phosphate buffers at pH 6, pH 7 or pH 8. Gels were pre-electrophoresed at 60 V, 10 mA for 30 minutes prior to loading samples (Garner and Revzin 1990). For holorepressor samples, the assay buffer and the gel solution contained 1 mM L-tryptophan, and the electrophoresis buffer contained 0.5 mM L-tryptophan. For experiments with the aporepressor, tryptophan was omitted from all components. The assay buffer was that used for the restriction-protection assay (Joachimiak et al. 1983). The DNA concentration (in duplex) was held fixed at different concentrations from 2 μM to 10 μM, and titrated with increasing concentrations of repressor. All retardation assays were repeated several times, using different preparations of protein, and in the case of *trpO*, three different preparations of DNA.

Competition assays were performed by mixing equal concentrations of *trpO* or *trpR* with *aroH* (typically 3 μM each), and titrating with increasing concentrations of holorepressor. As the electrophoretic mobility of the *aroH* fragment (22 base-pairs) is 10% less than that of *trpO* or *trpR* (20 base-pairs), the free DNA bands are resolved in these experiments, allowing the relative affinities for the two DNAs to be estimated.

To visualise the DNA, gels were stained with ethidium bromide for 10 min and then illuminated with UV light for photography on Polaroid film. In some cases, gels were stained with silver or Coomassie blue to visualise the protein. The protein barely enters the gel under the conditions of these experiments. Where binding was not stoichiometric, an estimate of the average affinity of the repressor for the DNA was made by considering the concentration of protein added at which the free and retarded bands are of approximately equal intensity. This is denoted $K_{0.5}$. Even allowing for the finite concentration of the DNA, $K_{0.5}$ is not always a true dissociation constant, as the final bound states are in some cases not precisely defined (see below).

Circular dichroism spectra were recorded on a JASCO J-600 spectropolarimeter. Titrations were performed by adding small aliquots of a concentrated solution of repressor to 2 μM DNA, all in the appropriate buffer, and scanning from 330 nm to 250 nm. Three repetitive scans were averaged to improve the signal-to-noise ratio. Molar ellipticities were calculated using the molar concentra-

tions of the operator fragments. The standard buffer for these titrations was 10 mM sodium phosphate, 100 mM KCl, pH 7.5. Tryptophan, when present, was 100 μM.

^{31}P NMR spectra were recorded at 30 °C on a Bruker WM200 instrument. The acquisition time was 2 s, and protons were decoupled using WALTZ 16. Concentrated trp repressor was added to the DNA solution to ensure that the DNA was initially in excess. One titration was carried out with trp holorepressor in the presence of 1 mM L-tryptophan, and a second was done with trp aporepressor, after which L-tryptophan was added.

Results

DNA *gel-retardation assay*

Figure 1A shows a typical DNA gel-retardation assay with the *trpO* sequence in the presence of tryptophan. As the concentration of repressor is increased, the intensity of the fastest moving band (free DNA) decreases, and a new band appears whose mobility, μ, relative to the free DNA band is 0.33 (Table 1). The free DNA band disappears when the concentration of protein is equal to that of the DNA, implying a stoichiometry of 1:1. This result was independent of the buffer system used and of the pH in the range 6 to 8. The stoichiometric binding indicates that the dissociation constant under these conditions is $\ll 2$ μM (the concentration of the DNA), and agrees with previous CD titrations (Lane et al. 1987). At higher concentrations of protein, the intensity of the band at $\mu = 0.33$ diminishes, and a new band appears at $\mu \approx 0.08$, which suggests that higher-order complexes can be formed unter these conditions. The band at $\mu = 0.33$ disappears completely when the ratio of protein to DNA exceeds 2:1, indicating that the more retarded band forms with a stoichiometry not greatly in excess of 2:1. In the absence of tryptophan, no band appeared at $\mu = 0.33$ at any point in the titration, but only an ill-defined smear appears near to the origin (not shown). This suggests that the band that appears at $\mu = 0.33$ in the presence of tryptophan corresponds to a specific trp holorepressor-operator complex. However, the disappearance of the free DNA band in the absence of tryptophan is not stoichiometric. From the concentration of repressor that was added to give equal intensities for the free and retarded DNA bands, and the total concentration of DNA, we estimate $K_{0.5}$ as ca. 5 μM, which is consistent with previous CD results on the aporepressor-operator complex (Lane et al. 1987). The hairpin form of *trpO* was not measurably retarded by repressor concentrations up to 5 μM even in the presence of tryptophan, indicating that this fragment of DNA has only very low affinity for the trp repressor.

The titration of *trpO*^{c} in the presence of tryptophan is shown in Fig. 1B. No band is observed at $\mu = 0.33$, but rather a strongly retarded band is present at $\mu \approx 0.08$. At still higher concentrations of repressor, only ill-defined bands are present close to the origin of the gel. The disappearance of the free DNA band of *trpO*^{c} (at 2 μM) is not complete until at least 13 μM holorepressor (Fig. 1B), consistent with an apparent dissociation constant in the μM range, as previously determined by CD (Chandler and Lane 1988). In contrast, the intensity of the free DNA band of *trp*MH disappears essentially linearly with increasing concentration of repressor in the presence of

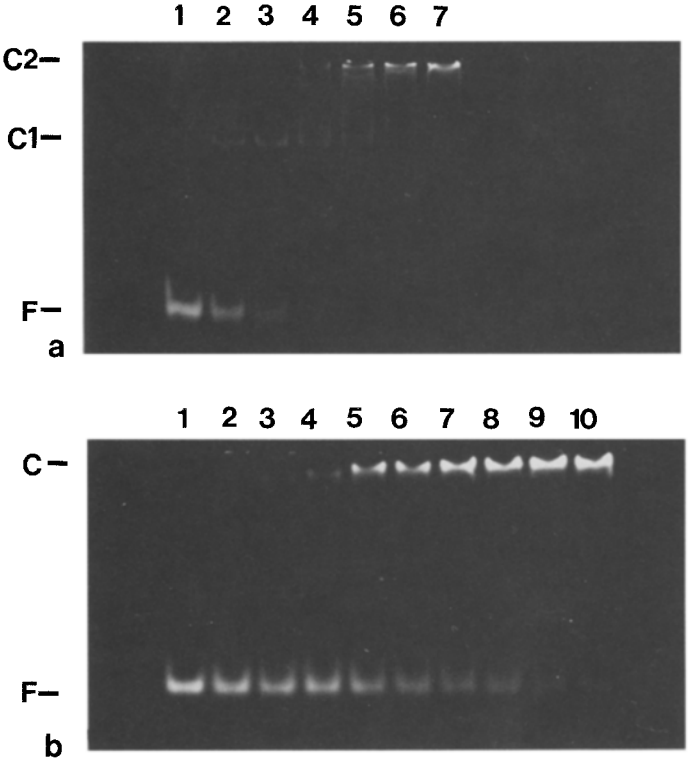

Fig. 1a, b. DNA gel retardation assays with the trp repressor. Gels were run as described in the methods. The buffer was TBE. **a** *trpO* DNA (2.2 μM) plus increasing concentration of trp repressor in the presence of L-tryptophan. The protein concentrations (μM) were Lane 1: 0, 2: 0.55, 3: 1.1, 4: 2.2, 5: 3.3, 6: 5.0, 7: 6.7. F shows the position of the free DNA, C1 the position of the first DNA-repressor complex and C2 the position of the second DNA-repressor complexes. **b** *trpO*^{c} DNA (2.2 μM) plus increasing concentrations of trp repressor in the presence of L-tryptophan. The protein concentrations (μM) were Lane 1: 0, 2: 1.1, 3: 2.2, 4: 3.3, 5: 5.0, 6: 6.7, 7: 8.3, 8: 10, 9: 11.6, 10: 13.3. F shows the position of the free DNA and C the position of the DNA-repressor complexes.

Table 1. Electrophoretic mobilities and CD data for trp repressor-operator complexes. μ: the electrophoretic mobility of the complex normalised to that of the free DNA. f: CD of repressor:DNA complex/CD of free DNA at 275 nm. The CD in the complex was taken at stoichiometric concentrations of DNA and repressor, and corrected for the weak CD of the protein

Operator (base-pairs)	μ	Apparent stoichiometry			Apparent $K_{0.5}$ (μM) at	
		gel	CD	f	10 mM KCl	200 mM KCl
trpO (20)	0.33, 0.08	1, ≥2	1	1.7	≪2	≪2
trpO^c (20)	0.08–0.1	nd[a]	nd	1.6	2	4
trpR (20)	0.33, 0.07	(1)[b]	nd	1.7	(<1)[b]	<1
aroH (22)	0.18, 0.08	1, ≥2	1	1.8	≪2	nd
*trp*MH (18)	0.09	≈2	≈2	1.3	<2	2

[a] Binding not stoichiometric at 2 μM DNA
[b] Smeared band

L-tryptophan (data not shown). Further, complete disappearance of the free *trp*MH DNA band occurred only at protein:DNA ratios of approximately 2:1 (Table 1). Raising the concentration of either *trpO*^{c} or *trp*MH to 10 μM still yielded no band at $\mu = 0.33$ when the [repressor]:[DNA] ratio was <1. Clearly, the hydrodynamic properties of the complexes of the holorepressor with *trpO*, *trpO*^{c} and *trp*MH are markedly different.

In titrations of *trpO*^{c} and *trp*MH in the absence of L-tryptophan, free DNA remained even at repressor concentrations of 10 μM. This indicates a similar non-specific dissociation constant of ca. 10 μM for each aporepressor-operator complex. This result can be compared with the value of $K_d = 13$ μM determined for non-specific binding using the alkaline phosphatase assay (Marmorstein et al. 1991).

The *aroH* operator shows high sequence homology with the *trp* operator, and those bases that are most sensitive to mutation, namely CTAG (Bass et al. 1987), are identical between the two sequences (see Methods). Gel retardation assays with *aroH* in the presence of L-tryptophan showed the formation of a retarded band at $\mu = 0.18$, which was formed with a 1:1 stoichiometry (data not shown), and which is consistent with $K_{0.5} \ll 2$ μM for the holorepressor-*aroH* complex. Addition of a two-fold excess of protein caused this band to disappear, with the formation of highly retarded complexes at $\mu \approx 0.08$. This behaviour is similar to that observed with the *trp* operator, and suggests that the 1:1 holorepressor-*aroH* complex is also stable. However, the relative electrophoretic mobility suggests that this complex may have somewhat different hydrodynamic properties compared with the holorepressor-*trpO* complex. In the absence of L-tryptophan, the free DNA band disappears with increasing concentration of protein, without the appearance of the band at $\mu = 0.18$. From the concentration dependence of the dissappearance of the free DNA band, a $K_{0.5}$ of ca. 2 μM was estimated.

In the competition assay between *aroH* and *trpO* (see methods) where the total concentration of protein is equal to half the total concentration of DNA, retarded bands corresponding to both complexes are observed (i.e. at $\mu = 0.33$ and $\mu = 0.18$, data not shown), with concomitant diminution of the intensity of the free DNA bands. Hence the affinity of *trpO* for the repressor is similar to that for *aroH*. We estimate that the relative dissociation constants must be within about a factor of three, in agreement with the results of Klig et al. (1988).

Titrations of *trpR* operator with trp holorepressor gave a weak, diffuse band at $\mu = 0.33$ at repressor:DNA ratios of less than one, accompanied by smearing. At higher concentration of repressor, only the highly retarded band at $\mu \approx 0.08$ is observed. This indicates that the affinity of the repressor for the *trpR* operator is significantly lower than that for *trpO*. This conclusion was confirmed by using a competition assay as performed for *aroH* versus *trpO* (see above). We found that the *aroH* band preferentially disappeared, with the band at $\mu = 0.18$ appearing while the free *trpR* band decreased in intensity only at higher concentrations of repressor. It is possible that the affinity of the repressor for the *trpR* sequence is dependent on context, and that when embedded in a long fragment of DNA, may bind the repressor substantially more tightly (Klig et al. 1988).

We have used an Hae III digest of pBR322, which contains fragments of length 8 to 587 base-pairs. The mobility of the fragments in this length range decrease nearly linearly with the logarithm of the molecular weight (or number of base-pairs). The retarded complex of the holorepressor with *trpO* at $\mu = 0.33$ is equivalent to a DNA duplex of about 180 base-pairs, or alternatively, the observed mobility is about half that expected from the molecular weight of the complex.

Circular dichroism

The advantages of optical techniques such as CD over the gel-retardation method are that the solution conditions can be readily manipulated, and the titrations can be analysed quantitatively. Although CD provides no information about the size of the complexes that are formed, it gives additional information concerning changes in conformation of the interacting components.

The CD spectra of all of the operator fragments are essentially conservative, and have extrema at wavelengths typical of B-DNA (Ivanov et al. 1973). The CD spectrum of the trp repressor in this wavelength range is very weak (Lane et al. 1987, Chandler and Lane 1988), and is <10% of that of an equimolar concentration of operator. The binding of the holorepressor to *trpO* and *trpO*^{c} increases the intensity of the circular dichroism at 275 nm by about 60% (Lane et al. 1987, Chandler and Lane 1988). This intensification of the near ultraviolet band probably arises from a change in the CD of the DNA. Binding to the *aroH* operator has a similar but larger effect (Fig. 2A). The increase in intensity at 275 nm is accompanied by only small changes between 230 and 260 nm. Under these conditions, the repressor binds to the *aroH* fragment tightly with a stoichiometry of 1:1 (see above). Similar spectral changes were observed with *trp*MH at low concentrations of salt (data not shown).

Titration data for the *trp*MH and *trpO* sequences are shown in Fig. 2B. In the presence of tryptophan, the titration curves show a break for both sequences. For *trpO* the break occurs when the concentration of repressor is equal to that of the operator (2 μM), whereas for *trp*MH, the break occurs when the repressor concentration is approximately twice that of the DNA concentration (4 μM), indicating a binding stoichiometry of 2 repressor dimers to one DNA duplex. At higher concentrations of salt (500 mM KCl), the repressor binds more weakly to *trp*MH, and becomes undetectable for *trpO*^{c} (Chandler and Lane 1988), while having no appreciable effect for *trpO* at these concentrations. This indicates that the affinity of the holorepressor for *trpO*^{c} and *trp*MH fragments is lower than for *trpO* and *aroH*.

The specificity of the interaction of the trp repressor with DNA was tested further by monitoring titration in the absence of tryptophan or in the presence of the antirepressor indole-3-acrylate (Marmorstein and Sigler 1989). The aporepressor caused only small changes in the CD

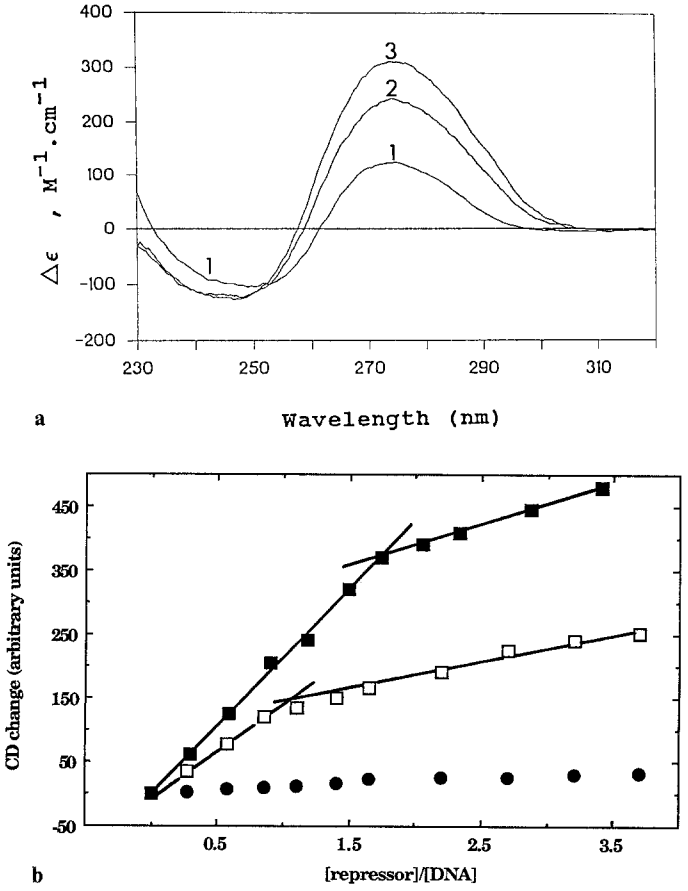

Fig. 2a, b. CD spectra and titrations of operator fragments with the *trp* repressor. CD spectra were recorded at 25 °C as described in the Methods. The buffer was 20 mM sodium phosphate, 100 mM KCl, 100 μM L-tryptophan, pH 7.5. **a** CD spectra of *aroH* in the presence of trp repressor at protein:DNA ratios of 0 (1), 2 (2) and 3 (3). **b** CD titrations of *trp*MH and *trpO* with the *trp* repressor. (■) 4 μM *trp*MH + 100 μM L-tryptophan, (□) 2 μM *trpO* + 100 μM L-tryptophan, (●) 2 μM *trpO* without tryptophan.

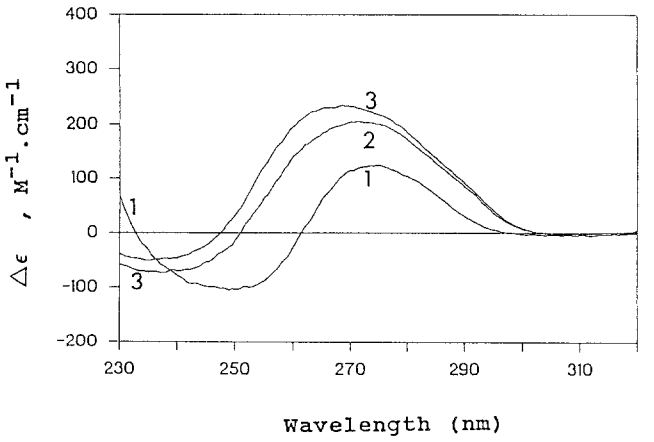

Fig. 3. Effect of trifluoroethanol on the CD spectrum of the *aroH* operator. Spectra of *aroH* in 0 (1), 80 (2) and 86% (3) trifluoroethanol. The buffer was 20 mM sodium phosphate, 20 mM KCl, pH 7.5.

intensity of the DNA fragments, and binding was completely abolished by the antirepressor. This indicates that tight binding depends on the presence of L-tryptophan, and that the increase in circular dichroism is absolutely dependent on the formation of repressor-DNA complexes. The size of the CD change as well as the affinity depend on the nature of the operator fragment (Table 1). Titration of *trpO* with trp holorepressor is accompanied by a large increase in the CD signal until saturation is achieved, i.e. stoichiometric binding (Lane et al. 1987). Further addition of protein causes a smaller increase in the CD signal (c.f. Fig. 2B). This secondary increase, which is larger than can be attributed to the CD of the holorepressor, and may reflect the formation of the non-specific complexes of higher protein:DNA stoichiometries observed in the gel retardation experiments (see above). CD titrations of the trp holorepressor with the different operator fragments have confirmed the results obtained by electrophoresis. For example, *trpO* and *aroH*, which produce complexes of 1:1 stoichiometry on gels, also show 1:1 stoichiometric binding in the CD titrations. Apparent affinities determined from the decrease in the intensity of the free DNA band on gels also agree with those determined by CD where the binding is sufficiently weak to be measured (Table 1).

The increases in CD of the DNA fragments on binding the trp repressor are reminiscent of those observed for the B to A transition induced by addition of alcohols (Sprecher et al. 1979). The addition of trifluoroethanol to 83% to the *aroH* DNA sample leads to a substantial intensification (ca. 80%) and broadening of the CD band at 275 nm, with a slight shift to 268 nm (Fig. 3); this behaviour is qualitatively similar to the effects of addition of the repressor (Fig. 2A), and suggests that the repressor may induce changes in the DNA similar to those produced by dehydration. The broadening of the positive band at 275 nm may account for some of the apparent shift of the negative band.

^{31}P NMR *spectroscopy*

The ^{31}P NMR signals report the conformation of the phosphate backbone (Gorenstein et al. 1991), and therefore can be used to monitor possible changes in backbone structure on forming a complex with a protein. Figure 4 shows the ^{31}P NMR spectrum of the *trpO* fragment in the absence and presence of the trp repressor. As expected, the spectrum of the free DNA is poorly resolved, consisting of 19 phosphate resonances within a shift range of only 0.66 ppm. On addition of the trp aporepressor, the intensity decreases and is accompanied by line broadening, but there are no obvious shift changes. We have already shown by gel-electrophoresis (see above) that the aporepressor forms complexes with *trpO* that have stoichiometries of at least 2 repressor dimers (M_r = 25 000) to 1 DNA duplex (M_r = 12 000). A complex of 2:1 stoichiometry would therefore have M_r = 62 000, and the phosphate line-widths would be expected to be at least five times as large as in the free DNA. In the presence of tryptophan, however, additional peaks appear at −17.5, −19.5, and −21.5 ppm, whose relative areas are 2:2:1. These peaks appear irrespective of the order of addition of tryptophan, but are critically dependent on

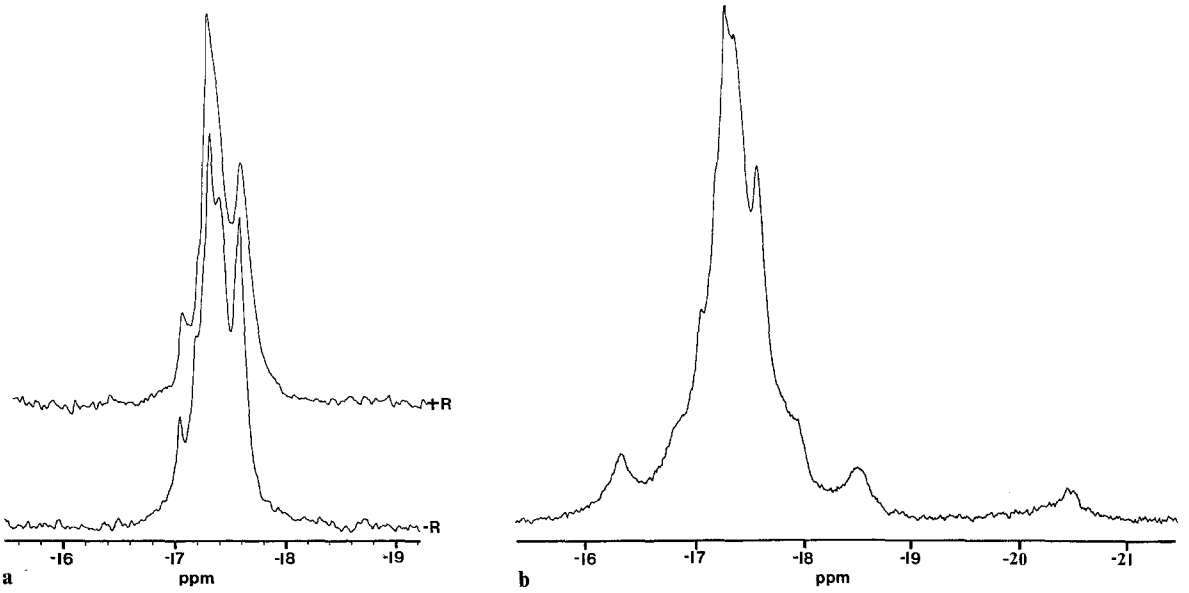

Fig. 4a, b. ^{31}P NMR spectra of the trp operator and complexes with the trp repressor. NMR spectra were recorded at 4.7 T and 303 K as described in the Experimental section. Free induction decays were multiplied by a 2-Hz line-broadening exponential before Fourier transformation, and referenced to external methylene diphosphonate. **a** *trpO* DNA (500 μM) ± apo repressor (400 μM) **b** *trpO* DNA (700 μM) + repressor (700 μM) + L-tryptophan (1500 μM).

the presence of the corepressor. Further, these peaks appear when the DNA is in excess of the protein, under which conditions, the complex has a stoichiometry of 1:1 (see above). The resonance width of the 1:1 complex of the trp repressor: *trpO* complex would be expected to be approximately three times that of the free DNA. Similar shifts were also obtained with the *aroH* operator (data not shown) in the presence of L-tryptophan. Electrophoresis of an aliquot of the samples used for NMR showed the characteristic retarded band at $\mu = 0.33$ in the presence of tryptophan (not shown), which indicates that the dominant species present in the NMR tube was the 1:1 holorepressor-operator complex.

Discussion

The electrophoresis experiments show that at low ionic strengths, even 20-base-pair fragments can bind more than one repressor dimer. The initial 1:1 complexes formed between the trp holorepressor and *trpO* or *aroH* decay on addition of more repressor, to produce complexes whose mobility is that of one containing at least two molecules of repressor bound to each molecule of operator DNA. The weaker-binding operator fragments, *trpO*^{c} and *trp*MH, form only complexes of at least 2 repressor molecules to 1 DNA, even when the DNA concentration is raised to 10 μM, and is in large excess over the protein concentration. This is equivalent to strong positive cooperativity, in which the first dissociation constant is larger than around 10 μM, and the second is much smaller. This result for the *trp*MH sequence agrees with recent findings by Carey et al. (1991) and Haran et al. (1992). The formation of the higher molecular weight complexes seems to be enhanced by the conditions of low salt concentration, especially when the pH is near the isoelectric point of the repressor. For example, Carey (1988) found extensive non-specific binding to 90 base-pair DNA fragments at repressor concentrations around 0.1 μM, whereas at near physiological concentrations of salt, the non-specific binding is much weaker (Marmorstein et al. 1991, Chandler and Lane 1988, and see above).

We have previously reported that the mobility of the 1:1 trp holorepressor: *trpO* complex is not dependent on the nature of the corepressor (Borden 1991). However, the different apparent sizes of the various complexes that are formed are dependent on the nature of the DNA target, particularly the 1:1 complexes or the holorepressor with *trpO* and *aroH*. This indicates that the nature of the complexes may be significantly different. Whether this has relevance to the possible protein-protein interactions implied by the tandem binding proposed by Kumamoto et al. (1987) remains to be determined with longer fragments of DNA.

The weak affinity observed in the absence of tryptophan for all DNA sequences (i.e. $K_{0.5}$ from ca. 2 to ca. 10 μM) may reflect a non-specific binding mode, that varies somewhat with sequence. Marmorstein et al. (1991) determined apparent non-specific dissociation constants of 13 to 21 μM: our results are very similar. However, as these complexes appear to have indeterminate stoichiometries, the true dissociation constant is not known. It may be significant that in the absence of tryptophan, and at low ionic strength, the trp repressor appears to form tetramers (Fernando and Royer 1992).

If weak binding and the formation of highly retarded bands containing complexes having protein:DNA stoichiometries $\geq 2:1$ is characteristic of non-specific binding, then the *trp*MH sequence proposed by Staacke et al. (1990) would have to be considered as a non-specific site.

It is clear from the CD data that there are significant changes in the conformation of the DNA on forming the specific complexes. The large increases in CD intensity are absolutely dependent on the presence of tryptophan, and appear to be of greatest magnitude for *trpO* and *aroH*. The intensification of the longer wavelength band and the near independence of the shorter wavelength band is similar to the effects of trifluorethanol, which produces a B to A transition (Sprecher et al. 1979). The changes associated with this transition have been attributed to the increased base-pair tilt in the A-form (Sprecher et al. 1979), though other conformational adjustments that change the base-base stacking could also increase the CD. Our results suggest that the binding of the trp repressor may cause a similar change in the relative orientations or stacking of at least some of the base-pairs. A change in base-pair tilt angles, or other helical parameters, is reflected in changes in backbone torsion angles, which, as Gorenstein and coworkers have argued, can have substantial effects on the ^{31}P NMR chemical shifts (Gorenstein et al. 1991). Clearly, in the presence of tryptophan, there are large changes in chemical shift of at least three phosphate resonances, in both the *trpO* and the *aroH* operators. This may be related to changes in the backbone torsion angles, though effects of solvation or formation of salt-bridges cannot be ruled out (Gerothanassis et al. 1991).

According to NMR data, the conformation of the 8 nucleotides involved in sequence-specific interactions with the repressor are in the range typical of B DNA in solution, i.e. glycosidic torsion angles anti and sugar puckers near C2′-endo (Lane 1991a, b), which presumably accounts for the B-like CD spectrum (see above). However, an x-ray diffraction analysis of an octamer containing the functionally important CTAG subsequence crystallised from methanepentanediol (Hunter et al. 1989) was predominantly in the A conformation. It is possible that the repressor stabilises a conformation similar to that found in the crystal state. This would be consistent with the x-ray structure of the repressor-operator complex (Otwinowski et al. 1988) which indicates that while the nucleotides are in the 'B' conformation, some of the helical parameters in the CTAG segments have A-like characteristics, particularly the large positive slide. Both the CD data and the NMR data are consistent with significant changes in the conformation of the DNA on forming specific complexes.

These results also show that well-defined complexes of 1:1 stoichiometry that represent specific repressor-operator complexes can be formed with short oligonucleotides, under near-physiological conditions of pH, ionic composition and temperatures, which is of considerable value for structural analysis both by crystallography and NMR.

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