Mutagenesis supports water mediated recognition in the trp repressor – operator system

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High resolution crystallographic analysis of the trp repressor – operator complex indicates that the principal determinants of specificity are water mediated hydrogen bonds between the helix – turn – helix and the identity elements of the operator. One such hydration site involves a conserved G-C base pair (designated G6) six nucleotides away from the dyad which, if changed symmetrically to any other pair (e.g. G6 – A) reduces affinity to non-specific levels. This same water site also contacts the conserved A5 which, if changed to G (mutation A5 – G), also diminishes affinity. The stereochemistry of the water mediated hydrogen bonding system predicts that the severe deterioration of in vitro binding caused by G6 – A should be reversed by a second deleterious mutation A5 – G. This proved to be the case. No other second mutation at conserved operator position 5 or 7 (flanking the G6 – A) reversed the effect of G6 – A. 

Key words: gel-retardation analysis/hairpin constructions/second site operator revertant/trp operator mutations/trp repressor – operator binding

Introduction

The trp repressor of Escherichia coli represses transcription of genes related to tryptophan biosynthesis by binding specifically to the conserved operator sequences in the promoter of four operons (trp, aroH, trpR and mtr) (Gunsalus and Yanofsky, 1980; Heatwole and Somerville, 1991). The protein is a dyad symmetrical dimer in which each subunit contains a helix – turn – helix motif that, in the presence of L-tryptophan, interacts specifically with the surface of successive major grooves of its pseudo-symmetrical target DNA, the trp operator (Kelley and Yanofsky, 1982; Schevitz et al., 1985). When two L-tryptophan molecules bind symmetrically to the aporepressor (one per subunit), the two symmetrically disposed DNA binding motifs are oriented so that they support strong specific binding (Otwonowski et al., 1988). The crystal structure of the trp repressor – operator complex revealed that the ‘recognition’ of the operator by the repressor is largely the result of stereospecific solvent mediated interactions between the repressor and the bases that specify the operator’s identity (Otwonowski et al., 1988; R.-G.Zhang, C.K.Kundrot, Z.Otwonowski and P.B.Sigler, submitted). The region of the repressor involved in these water mediated contacts is at the N-terminus of the second helix of the helix – turn – helix motif (Figure 1). Here we report evidence from in vitro mutagenesis experiments that supports the participation of water molecules in trp repressor – operator recognition. The crystal structure of the repressor – operator DNA complex was used to predict and design a second site revertant of a severely defective operator mutation; that is by changing a second base pair (symmetrically) we reversed the effects of the original operator mutation.

Sequence consensus and mutagenesis experiments (Bennett and Yanofsky, 1978; Bass et al., 1987; Staacke et al., 1990) clearly indicate that the base pairs at positions 4, 5, 6 and 7 from the dyad are the strongest determinants of the operator’s identity. Of these, base pair 6 appears most important; it is absolutely conserved in all E.coli operators (Figure 2A) and mutation to any other base pair reduces the stability of the repressor – operator complex by at least 102-fold in the in vivo challenge assay of Youdarian and coworkers (Bass et al., 1987). The base pair at position 5 from the operator’s dyad is also conserved, but in vivo mutagenesis indicates that a replacement of A5 with G is better tolerated (only 1.7-fold diminished) than a G5 to A transition at position 6. Like position 6, a pyrimidine at position 5 results in a complete loss (102-fold) of specific affinity in vivo (Bass et al., 1987). The stereochemistry underlying the importance of G5 and A5 as identity elements is illustrated in Figure 1. In the crystalline complex, both of these purines interact with the protein through water mediated contacts. In this paper we will focus on the water site that bridges the amide NH of Ala80 to G5 and A5 through a stereospecific tetrahedral arrangement of hydrogen bonds.

The premise of this paper is as follows: if the water mediated hydrogen bonds seen in the 1.9 Å crystal structure (R.-G.Zhang, C.K.Kundrot, Z.Otwonowski and P.B.Sigler, submitted) are important in producing strong sequence-specific interactions between the trp repressor and operator, then inspection of Figure 1 would suggest that the deleterious effect of replacing G6 with A6 might be reversed if A5 were replaced by G5. This second site reversion simply swaps the donor and acceptor functions at the 6 position of these two critical purines with preservation of the tetrahedral hydrogen bonding scheme at the bridging water molecule. Using a variation of the gel-mobility retardation assay developed by Haran et al. (1992) and a competitive filter-binding assay based on the study of Hurlburt and Yanofsky (1990) we tested this hypothesis and found the predicted reversion.

Results

A double mutation of the trp operator restores specific repressor binding

To test the predictions stated above, a set of DNA oligonucleotides was synthesized containing an idealized wild type
operator sequence and variants containing one or two pairs of symmetrical changes (Figure 2). Repressor affinity for these oligonucleotides was measured by gel-retardation and filter-binding assays (Figure 3). Symmetrical 18 bp test sequences were incorporated into the 21 bp stem of a generic 'hairpin' construction (Figure 2) similar to that introduced by Haran et al. (1992) in the binding experiments that confirmed the true trp operator sequence. Such hairpin constructions prevented the dissociation of short two-stranded palindromic duplexes (20 bp or less) and, thereby, eliminated a competing equilibrium which confounded a previously reported in vitro binding analysis (Staacke et al., 1990). This competing equilibrium arises from the fact that the stability of a short conventional two-stranded duplex is bimolecular in the concentration of the component strands. At the nanomolar (or lower) concentration of DNA usually used in in vitro binding assays, there is an appreciable dissociation of the individual strands of two-stranded 'palindromic' duplexes. Once dissociated, each strand can fold back on itself to form a small hairpin whose stability is concentration independent. Thus low concentrations (or passage through the gel) favors dissociation of such duplex targets. Tethering the ends of the palindromic duplex strands together with a 5 bp loop converts the construct into a hairpin (Figure
operator mutation restores water mediated interaction with trpR

2). This obviates the second equilibrium and makes feasible the use of short symmetrical targets at the low concentrations required for accurate quantitation of high affinity sequence-specific binding. The five nucleotide loop of the hairpins used here apparently does not interfere with repressor binding when separated by 2 bp from the 18 bp target in the stem of the hairpin (Haran et al., 1992).

Hairpin constructions bearing symmetrical single and double mutations at positions 5, 6 and 7 of the operator sequence were electrophoresed in the presence of trp repressor (Figures 2 and 3). Figure 3A shows the effect of single (symmetrical) mutations at position 5 of the operator on repressor-retarded gel electrophoretic mobility. The only symmetrical mutation at this position which results in a measurable band shift is $A_5 \rightarrow G$. Although the affinity of the repressor towards the operator carrying this mutation is easily detected by a retarded band, it is diminished to 8% of the wild type target affinity when quantitated by the filter-binding experiments described below. Any other base substitution at position 5 diminishes affinity to the point where we cannot detect repressor-DNA complexes in the gel-shift assay even at repressor concentrations as high as 1 $\mu$M (Figure 3A and data not shown). These results are fully consistent with the results of the in vivo challenge assay (Bass et al., 1987). On the other hand, any mutation at position 6 completely abolishes repressor binding [Figure 3B and in vivo analysis of Bass et al. (1987)]. In the absence of stereochemical reasons to the contrary, these results with single (symmetric) operator mutations at sequence positions 5 and 6 would cause one to predict that any combination of symmetrical double mutations at positions 5 and 6 would lower the affinity for the repressor beyond that observed for mutation at position 6. However, as outlined above, the crystal structure of the trp repressor-operator complex identifies the symmetrical double mutation that changes both $G_6 \rightarrow A$ and $A_5 \rightarrow G$ should restore specific repressor binding. Figure 3 shows that this symmetrical double mutation does, indeed, restore a level of binding to the operator that is easily detected by the gel-shift assay. Although the affinity is reduced to 5.7% of wild type it represents an essentially complete reversion of the devastating effect of the $G_6 \rightarrow A$ mutation. We tested operators carrying all other double mutations at positions 5 and 6 as well as at positions 6 and 7 (Figures 2 and 3), and none of them can restore detectable repressor binding in the gel-retardation assay. In fact, all such double mutations, when quantitated by filter-binding studies, show an affinity the same as or lower than the $G_6 \rightarrow A$ mutation (Figure 4).

Quantitation of repressor affinity for mutant operators

We have used competition for nitrocellulose filter-binding to determine the relative affinities of the operator mutants. Figure 4 shows the results of competition between a radioactive 43 bp trp promoter fragment (Hurlburt and Yanofsky, 1990; Haran et al., 1992) and DNA hairpins containing the wild type and mutated trp operator targets. The hairpin containing an idealized wild type operator sequence competes best with the 43 bp promoter fragment; in fact, as shown by Haran et al. (1992), it competes as well as an unlabeled 43 bp promoter fragment. The symmetrical mutation $G_6 \rightarrow A$ reduces the affinity for the repressor >500-fold. However, a simultaneous change of $G_6 \rightarrow A$ and $A_5 \rightarrow G$ restores affinity nearly to the level seen with a single $A_5 \rightarrow G$ mutation (5.7% of the wild type). Although wild type affinity has not been completely restored, the severely deleterious effect of the $G_6 \rightarrow A$ mutation has been reversed.

Discussion

Structural basis for the predicted reversion of $G_6 \rightarrow A$

It is appropriate to employ site-directed mutagenesis to test mechanisms inferred from crystallographic studies. This is especially important when the inferences suggest novel and untested biochemical mechanisms. It is for this reason that this paper addresses the crystallographic finding of fixed water molecules as key elements in the stereochemistry of a specific protein-DNA interaction. These observations were unexpected and were challenged by gel-retardation assays that purported to show that an alternative sequence, and not the traditional trp operator used in the crystal structure (Figures 1 and 2a), is the target of the trp repressor (Staacke et al., 1990). Haran et al. (1992) refuted this assertion, showing that the gel-retardation results were anomalies arising from competing equilibria and, once these artifacts were recognized and experimentally addressed, gel-shift and filter-binding experiments confirmed the traditional operator sequence as being the trp repressor target. They
also pointed out that sequence consensus and mutagenesis support the alignment of the repressor on the operator seen in the crystalline complex. Most recently, the structures of the trp repressor—operator complex have been determined in two different crystal forms grown from high salt (Lawson and Carey, 1993; A.Joachimiak, Z.Otwinowski, R.Hegde and P.B.Sigler, personal communication). Both structures show the same interface as that described in the original X-ray study (Otwinowski et al., 1988), and thereby relieves the concern that the solvent of crystallization may have altered the chemistry of the specific interface (Brennan and Matthews, 1989; Carey et al., 1991).

**Water mediated contacts determine base preferences at positions 5, 6 and 7**
The water mediated interactions between the N-terminal end of helix E and A_{-7}, G_6 and A_5 provide a stereochemical rationale for the base preferences exhibited in the operator consensus sequence (Figure 2) and in mutagenesis experiments (Bennett and Yanofsky, 1978; Bass et al., 1987; Staacke et al., 1990). In earlier work, the requirement for a purine at operation position −7 was shown to be a water mediated contact between the γ-hydroxyl of Thr83 and the N7 of the purine (Otwinowski et al., 1988; Luisi and Sigler, 1990). The preference for A over G is due to the arrangement of neighboring polar groups that orient the water molecule so that it will only accept a hydrogen bond from the substituent at the 6 position of the purine. Thus, a hydrogen bond donor, the exocyclic amine of adenine, is preferred over the carbonyl O6 of guanine (Otwinowski et al., 1988). Here we narrow the focus to the water mediated complementarity between the exposed peptide NH groups at the very tip of the E helix (Ile79 and Ala80) and purines G_6 and A_5 (Figure 1). The water molecule at Ile79 receives two hydrogen bonds from the protein: one from the peptide NH of Ile79 and the other from the N terminus of Lys72. The special orienting role of the Lys72 is evident from the fact that other bacterial and phage regulatory

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**Fig. 3.** Gel-mobility retardation analysis of wild type and mutant operator sequences using hairpins whose stems contain the symmetrical duplexes shown in Figure 2. (A) The wild type trp operator is compared with single mutations at position 5. Mutations are indicated on the hairpin diagram shown above gels. DNA concentration is 1 nM in all experiments. The stoichiometry of the complex was shown previously to be one dimeric repressor bound to one DNA fragment (Haran et al., 1992). (B) The G_6 → A mutation plus all four possible base pairs at position 5. (C) The reversion of G_6 → A by A_5 → G (first three lanes) plus three examples of mutations flanking changes at position 6 that do not support gel band shift of the trp operator.
proteins with a helix-turn-helix have an Ala at the corresponding position, whereas mutagenesis shows that only Lys72 (or, marginally, the mutation Lys72 → Tyr) supports the function of the trp repressor. It is likely that this water molecule is highly polarized as it is fixed to the positive pole of the E helix’s dipole as well as the positively charged ammonium group of the Lys72 side chain. Thus, a firmly positioned and oriented water molecule donates a highly polarized hydrogen bond to the electronegative N° of the purine at position 6 of the operator. This means a purine is required at position 6 in the wild type operator, but adenine is not acceptable.

We observe here that changing G6 symmetrically to any other base, including A6, dramatically reduces the stability of the complex to ‘non-specific’ levels. This is consistent with the absolute conservation of G6 in all E.coli trp operators (Figure 2a) and with the results of in vivo mutagenesis experiments mentioned earlier (Bennett and Yanofsky, 1978; Bass et al., 1987; Saacke et al., 1990). The reason why G6 rather than A6 is required is clear from Figure 1. Tetrahedrally hydrogen bonded water donates two and accepts two hydrogen bonds. Since the amide NH at Ala80 and the exocyclic amine of A6 are both donors, the water fixed to the amide NH of Ala80 must donate a hydrogen bond to the 6 substituent of this purine. Thus, the O° carbonyl of G6 is strongly preferred to the exocyclic N° of A6. The loss of a hydrogen bond (two hydrogen bonds when the symmetrical site is considered) may not account for all of the differences in affinity between G6 and A6; the disruptive effect of A6 may be augmented by steric hindrance between the proton of the exocyclic amine and the firmly fixed water molecule at NH of Ala80. Steric hindrance can be especially disruptive to the complex if the participating groups are otherwise well fixed by their neighbors and the structure cannot adjust locally to accommodate the interference. This may be the case with purine 6 for three reasons. First, the DNA is tightly anchored to the protein through an extensive array of phosphate contacts; second, the purine at position 6 is sandwiched between base pairs that have ‘specific’ interactions with the protein; and third, the position of the water molecule is stabilized by three other interactions.

It is also clear from Figure 1 that a purine is required at position 5. Substituting a pyrimidine for A5 provides no basis for specific interactions and hence results in severe deterioration of binding (Figure 3A). The change A5 → G, which replaces the exocyclic amine with a carbonyl, also decreases affinity, but by only 10-fold, which is considerably less than the reciprocal change at G6. One explanation of the relative tolerance for O° in the purine at operator position 5 is that the range of hydrogen bond acceptance angles for the carbonyl group is much larger than for the amino group, thus providing the intervening water molecule more degrees of freedom to adjust. Also, when replacing an amine with a carbonyl, no hydrogen atoms are introduced to cause steric hindrance. In general, the hydrogen bonding patterns of carbonyl oxygens are less stereo-restrictive, as evidenced by the preference of oxygen over nitrogen atoms.
for surface hydration in proteins (Baker and Hubbard, 1984). Other factors unrelated to hydrogen bonding, such as sequence-dependent deformability, may also influence the affinity of the mutant operators. Clearly, a complete structural explanation for the disruptive role of any change in functional groups must await the crystal structure of the complexes containing mutationally altered operators.

The second site mutation: $A_8 \rightarrow G$ reverses the deleterious effect of $G_6 \rightarrow A$

We have shown that introducing a second symmetrical mutation, $A_8 \rightarrow G$, reverses the severe deterioration of specific binding caused by the symmetrical change, $G_6 \rightarrow A$. This result was predicted from the tetrahedral pattern of water mediated hydrogen bonds between the operator’s conserved purines at sequence positions 5 and 6 and the peptide NH at position 80. No other mutation at position 5 will revert the $G_6 \rightarrow A$ mutation; indeed, all other symmetrical double mutations involving positions 6 and 5 or 6 and 7 are reduced in their affinity for the repressor by a factor of at least 500.

We ascribe the reversion to the restoration of the water mediated hydrogen bonding pattern at the peptide NH of Ala80 where the polarity of the two hydrogen bonds has been reversed, i.e., an acceptor (the O$^\circ$ of guanine 6) has been replaced by a donor (the exocyclic N$^\circ$ of adenine 6). Conversely the donor, the N$^\circ$ of adenine 5, has been replaced by an acceptor, the O$^\circ$ of guanine 5.

Why has the reversion of the $G_6 \rightarrow A$ not fully restored wild type affinity to the operator? Although formally the hydrogen bonding system has been restored, its stereochemistry has not. The oxygen atom of the intervening water is much closer to the plane of the purine at position 5, i.e. considerably below the plane of purine 6. Since the directionality of hydrogen bonds from the exocyclic aromatic amine is more constrained to the aromatic plane, the hydrogen-bonds donated to the out-of-plane water by $A_8$ are weakened. The O$^\circ$ of $G_6$ is preferred, as noted earlier, because the carbonyl oxygen is more tolerant of out-of-plane interactions. Here again there may be additional factors in the ‘double mutant’ that alter the stability of the complex without affecting the hydrogen bonding scheme. A crystallographic analysis of the trp repressor complex containing this doubly altered operator is called for.

Materials and methods

Purification of trp repressor

The trp aporepressor protein was overexpressed in the strain pETG13apr3/BL21DE3 kindly provided by Dr C. Lawson (Brookhaven National Laboratory). The protein was purified using the procedure of Joachimiak et al. (1983). In an alternative accelerated purification protocol the protein was chromatographed on a Mono Q (16/10) column and eluted with a linear 100 – 300 mM NaCl gradient. This was followed by separation on a Mono S (10/10) column and the aporepressor was eluted with linear 0 – 250 mM NaCl gradient. Aporrepressor preparations were shown to be homogeneous by polyacrylamide gel electrophoresis under denaturing conditions and silver staining. The protein is fully active, upon addition of L-tryptophan, in DNA binding assays and in calorimetric titrations.

Preparation and labeling of DNA fragments

All oligodeoxynucleotides used in this study were synthesized on an automated DNA synthesizer (Applied Biosystems) using reagents from Peninsula. The oligonucleotides were purified by electrophoresis on polyacrylamide gels containing 7 M urea. DNA fragments were eluted from the gel with 0.5 M sodium acetate pH 5.2 and ethanol precipitated. Excess salt was removed by washing the precipitate with 70% ethanol and the DNA was vacuum dried.

Small amounts (2 – 4 pmol) of oligonucleotides were labeled with $^{32}$P or $^{35}$S. The 5' termini of hairpin duplexes (Figure 2) were labeled with $[\gamma-^{32}P]ATP$ (specific activity $\sim 5000$ Ci/mmole) and T4 polynucleotide kinase. The 3' recessed ends of the trpEDCBA promoter fragment used in filter-binding competition experiments (Figure 4) were filled in with $[\alpha-^{35}S]dATP$ (specific activity $\sim 600$ Ci/mmole), dTTP and the Klenow fragment of DNA polymerase I. The hairpinned radiolabeled oligonucleotides were purified by polyacrylamide gel electrophoresis in the presence of 7 M urea, while the trpEDCBA promoter was purified on native polyacrylamide gels. All fragments were extracted from the gel as described earlier. After precipitation the radiolabeled DNA samples were dissolved in buffer (10 mM Tris – HCl, 1 mM EDTA pH 7.6) and 5 mM dithiothreitol. Hairpin DNA was annealed by heating the DNA samples to 95°C and shock cooling on ice. All DNA samples were stored at $\sim 80°C$. DNA solutions of known concentration and specific radioactivity were prepared by adding the labeled DNA to a 50:1 molar excess of unlabeled DNA of known concentration (Haran et al., 1992).

Gel mobility retardation assay

The gels used in Figure 3 were native 16% polyacrylamide gels (acrylamide/bisacrylamide ratio = 75:1) cast and run with 10 mM phosphate buffer (pH 6.5) and 0.25 mM t-tryptophan. The gels were prerun at 12.5 V/cm for 1.5 h, and run at 25 V/cm for 3 h, with recirculation of the buffer. The gel temperature was kept at 15°C by using a constant temperature gel apparatus (Hoeffer) and a circulating refrigerated water bath (VWR). Samples (10 μl) comprising 1.0 μg DNA, 5 μM or 50 μM trp repressor, 0.1 mM phosphate buffer (pH 6.5), 0.5 mM t-Trp, 25 mM NaCl and 0.1% Nonidet P40 (Sigma), were incubated for 30 min at room temperature. Samples were loaded while the gels were running. After electrophoresis the gels were vacuum dried at 80°C. The bands were localized by autoradiography.

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