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Modulation of the *Escherichia coli* Tryptophan Repressor Protein by Engineered Peptides

Chris Fenton, Anders Hansen, and M. Raafat El-Gewely[1]
Department of Biotechnology, Institute of Medical Biology, University of Tromsø, MH-building,Tromsø 9037, Norway

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We have used the *E. coli* tryptophan repressor (TrpR) as a model protein for modulation by engineered peptides both *in vivo* and *in vitro.* The tryptophan operator promoter-lacZ reporter system was used to investigate the *in vivo* ability of several synthetic peptides to modulate TrpR function. GMSA (gel mobility shift analysis) was used to study the *in vitro* ability of the peptides to modulate binding of the TrpR protein to the operator DNA. Peptides WRW, DRW, DW, RW enhanced TrpR binding to the operator *in vivo* at 100 μM concentrations. The same peptides enhanced TrpR binding to the operator *in vitro* at 1 mM concentrations. The peptide RRW reduced TrpR binding to the tryptophan operator both *in vivo* and *in vitro.* Thus the peptide RRW acted more as an inducer than corepressor. The peptide WR could neither enhance nor impede binding between TrpR and the operator *in vivo* or *in vitro,* suggesting that the presence of a carboxyl tryptophan residue may be necessary for binding to the TrpR protein. Thin layer chromatography was used to ensure that the peptides had not been subject to proteolysis during the *in vitro* gel mobility shift assays. © 1998 Academic Press

Key Words: **gel retardation; DNA-protein interactions; peptides; regulation.**

Several characteristics of the *E. coli* TrpR protein make it an ideal candidate for the detailed study of the relationship between structure and function. TrpR regulates 5 unlinked operator regions *trpEDCBA*, *aroH*, *aroL*, *trpR*, and *mtr* (1–5). TrpR is highly conserved among enterobacteria (6) and is easily purified in large amounts from E. coli (7). The TrpR protein has been subject to intensive study over a 25 year period resulting in the elucidation of 6 crystal structures (8–13), over a dozen NMR studies (14–16), affinity constants for the tryptophan or tryptophan analogs binding to the aporepressor, and subsequent holorepressor binding to the operator (17, 18).

Before the TrpR protein can bind the *trp EDCBA* operator sequence and repress tryptophan synthesis, it must first be activated by the binding of two molecules of tryptophan. The corepressor tryptophan binds into the DNA binding pocket becoming an integral part of the DNA recognition face (8). Possible protein-protein interactions between adjacent TrpR dimers (11) or perhaps even between TrpR and other transcription factors (19) may also effect TrpR operator interactions.

In this paper we have succeeded in modulating TrpR activity using small synthetic peptides. Peptides offer a number of advantages for the study of a potential modulators, the most important of which is the active uptake of peptides into cells. Several di-and tri-peptide scavenger systems have already been characterized in *E. coli* (20–22).

We have synthesized peptides containing tryptophan residues in the hopes of targeting the flexible corepressor pocket. We have tested the ability of the engineered peptides to modulate the *trpOP-lacZ* fusion *in vivo* as well as tested their *in vitro* ability to induce TrpR binding to the natural 241 bp tryptophan operator DNA fragment by gel mobility shift analysis.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

All bacterial strains and plasmids used are outlined in Table 1.

Peptide Synthesis

Peptides were synthesized using Fmoc chemistry combined with a Milligen 9050 Plus Pepsynthesizer (Millipore Corp., Waters Chro-

[1] Corresponding author. Fax: 47 776 45350. E-mail: raafat@fagmed.uit.no.

Abbreviations: *trp OP, trp EDCBA* operator promoter; *cml,* chloramphenicol resistance gene; TrpR, *E.coli* tryptophan repressor protein; WRW, Peptide tryptophan-arginine-tryptophan; RW, Peptide arginine-arginine-tryptophan; RW, Peptide arginine-tryptophan; WR, Peptide tryptophan-arginine; DRW, Peptide aspartic-arginine-tryptophan; DW, Peptide aspartic-tryptophan; ONPG o-nitrophenyl-b-D-galactopyranoside; β-gal β-galactosidase.

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TABLE 1

Outline of Strains and Plasmids Utilised in Present Study

	Relevant characteristics	Source or reference
Strains		
CY 15070	Tna2, trpR2, LaqIq	(45)
CY 15075	W3110, tnaA2, ΔlacU169, trpR2(λTLF1), β-gal production under trpR regulation	(32)
XL1 Blue(R)	SupE44, hsdR17, recA1, endA1, gyrA56m relA1, lac−, F′(proAB+, LaqIq, lacZDM15, Tn10 tet)	Stratagene
K37	F^-, galK, Gm^R	D. Friedman, University of Michigan
Plasmids		
pWPY2	Amp^R, LacZ, 241 bp trp operator/promoter (parent pBluescript)	(19)
pJR2	Amp^R, tac P/O, trp leader RBS, trpR, rpoC (parent ptacterm)	(45)
pACYC-trpR	TrpR, Tet promoter, Cm^R, (parent pACYC184)	(46)
pACYC184	Cm^R, Tet^R, p15a origin	(47)
pTZ1	Amp^R, tac P/O, lacZ, (parent pOX21)	(48)

matography, Milford, MA). Peptides were purified using reverse-phase high performance liquid chromatography and analyzed on a Fast Icon Bombardment Mass Spectroscopy instrument (VG Analytical, Manchester, UK).

β-Galactosidase

Plasmid pACYC184-trpR in *E. coli* cell strain CY15075 was grown in a minimal E-media (23) supplemented with 25 μg/ml chloramphenicol, 0.2% glucose and 0.2% Difco Casamino acids (24, 25). Peptides were added in 100 μM amounts. β-galactosidase assay was performed according to Miller, 1972 (26) but with modifications that allowed us to measure activity in microtitre plates, modifications reported previously in (25). A 200 μl mixture of 20 μl cells and 180 μl modified Z-buffer was incubated at 30°C and ß-galactosidase activity was measured at 405 nm for ONPG with a Molecular devices Vmax microplate reader (Falcon 96-well flat-bottomed). Modified Z-buffer contained 0.1M phosphate-buffer, pH 7.3, 6 mM $MgCl_2$-$6H_2O$, 30 mM DTT, 0.15% CTAB, 4mg/ml ONPG. ONPG was added directly before use. We have noticed that CTAB the active lysing detergent in the Z-buffer proved to be a source of error. The presence of bubbles often made it impossible for the Molecular devices native software to correctly analyze the correct slope of the β-galactosidase reaction. The raw data was therefore transferred to an IBM compatible PC and the correct slopes were calculated using a small program that was written in Matlab. β-galactosidase was calculated according to the Lambert law using the extinction coefficient of ONPG (13,300 mol/cm). Correction was made for length of the light path in the microplate reader. Absorbency of cells was measured at 590 nm instead of 600 nm but corrected for. The assay was done in four parallels.

Gel Mobility Shift Analysis

Gel mobility shift analysis was essentially as described previously in Fenton and El-Gewely, 1997 (27).

Gel preparation. Same as (27) but 0.1 mM of the corresponding peptide was added to the gel matrix.

Incubation reaction. The incubation reaction (28), total volume 20 μl, contained 12.5 mM NaH_2PO_4 (pH 6.0), 25 mM NaCl, 0.5 mM tryptophan, or 1 mM peptides, < 5 pmol labeled DNA, 16% (vol/vol) glycerol, 0.01% xylene cyanol, and 0.01% bromophenol blue. 0.5 μg of non-competitive inhibitor poly[dI dC]·poly[dI dC] (Sigma Product number 9389) was included in the 20 μl incubation buffer. The incubation buffer contained 50 μM tetrasodium pyrophosphate in order to inhibit any phosphatase activity remaining in the protein extract (27). The reaction mixtures were allowed to equilibrate for 15 minutes at 37°C. Protein samples stored at −70°C were thawed in ice water before use.

Gel electrophoresis. Gels were run at 50 V, 1.5 mA for 40 Vhrs. Gels were run at 50V, 1.5 mA for 5 minutes before sample application.

Gel analysis. The gels were dried on to Whatman no. 1 paper at 85°C for 45 minutes using a Biorad 583 GelDryer and then put in a phosphostorage cassette overnight (Molecular Dynamics, 880 E. Arques Ave, Sunnyvale CA 94086) . The gels were scanned by Molecular Dynamics phosphoimager 400e and later analyzed by the image quant program.

Northern Analysis

CY15070 cells containing the pACYC-trpR plasmid were grown in 3ml of E-media containing the 100μM of the corresponding peptide at 30 C until a A600 of 0.8 was reached. RNA was isolated according to (29) and subjected to electrophoresis through a 1% formaldehyde gel (30). The gel was capillary blotted onto a nytran filter (Schleicher and Shuell, D-37582 Dassel, Germany) filter using standard protocols. RNA was UV cross linked by 45 seconds of UV exposure. The filter was pre-hybridized for 3 hours at 65°C and then hybridized for 36 hours at 65°C. Hybridization buffers were in accordance with Sambrook, 1989 (30). The filter was dried at room temperature and placed in a phosphoimager cassette overnight. The filter was then scanned by Molecular Dynamics phosphoimager 400e and later analyzed by the image quant program.

The 1.7 kb double stranded DNA containing the *lacZ* gene was cut from plasmid pTZ1 using BamH1 restriction enzyme. The 1.3 Kb ds DNA containing the *cml* (chloramphenicol resistance gene) was cut from pACYC184 using restriction enzymes EcoRI and AvaI. Both fragments were labeled with Pharmacia ready-to-go DNA labeling kit (dCTP) according to manufactures instructions. (Pharmacia S-751 82, Uppsala, Sweden) . Both ds DNA fragments were then subjected to two rounds of phenol-chloroform extraction followed by ethanol precipitation and washing the pellet in 80 percent ethanol before being added to the hybridization buffer.

Thin Layer Chromatography

The thin layer chromatography method used was that of Degterev, 1996 (31). TCL aluminum sheets silica gel 60 W without fluorescent indicator (Merk, Darmstadt) were washed briefly in

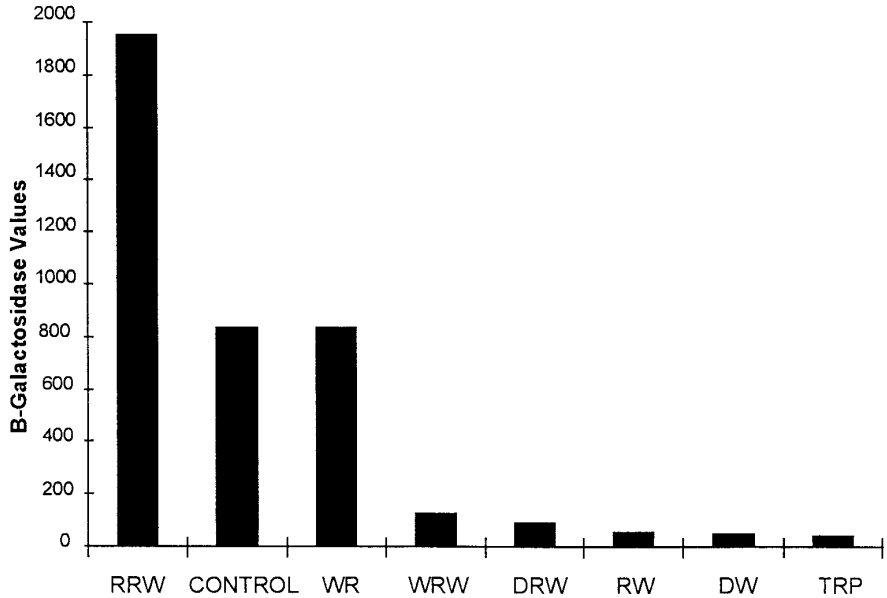

FIG. 1. Peptide regulated β-galactosidase transcription from the tryptophan operon lacZ reporter system. E. coli strain CY15075 containing a chromosomal LacZ under the control of Trp operator in E-media shows a wide range of peptide dependent activity. The TrpR protein is constitutively expressed from the pACYC-trpR plasmid tet promoter. Distilled water was used as a control.

methanol/chloroform (1 + 1, v/v) and dried for 20 minutes at room temperature to remove impurities. A glass tank was saturated by lining the tank with filter paper soaked in the mobile phase for two hours before the experiment. The ten 1.5 cm lanes were marked with a standard pen. Half of the samples were prepared as described in the gel retardation incubation reaction. The other half were prepared in an identical manner with water substituted for the TrpR protein extract. The mobile phase was 2-propanol-25% aqueous ammonia (7 + 3, v/v) and plates were developed to a distance of 7.0 cm. Developed plates were dried at room temperature for 20 minutes and then heated a 110°C for 30 minutes. Peptide samples were detected by dipping in a ninhydin solution (ninhydrin 300 mg, acetone 97ml, acetic acid 3ml). After dipping the plates were left to stand for 10 minutes and then dried at 70°C for 10 minutes. Notice that the ninhydrin spots fade with time. The plates were then scanned and analyzed for spot intensity after background correction by Matlabs Image Processing Toolbox.

RESULTS

β-Galactosidase

The addition of 100 μM concentrations of peptides (RW, DW, DRW) to the tryptophan operator-LacZ reporter system in E-media resulted in β-galactosidase values under 50 units (Figure 1). The addition of 100 μM WRW gave a slightly higher β-galactosidase value, 122 units. In contrast the addition of WR to the media gave the same result as the control (an equal volume of distilled water) 800 units. Surprisingly the addition of RRW gave a β-galactosidase values of approximately 1800 units or 2-3 times higher than the control. The addition of RRW, tryptophan or water to unmodified pACYC184 vector (no *trpR*) gave equal β-galactosidase values of about 7000 units. Therefore the cloning vector pACYC184 without the *TrpR* sequence could not be differentially regulated by either tryptophan, RRW, or water (results not shown).

Northern Analysis

Results from a northern analysis autoradiogram are shown in Figure 2. The lower control band represents the level of constitutively transcribed c*ml* transcript. The upper band represents the peptide-regulated *lacZ*

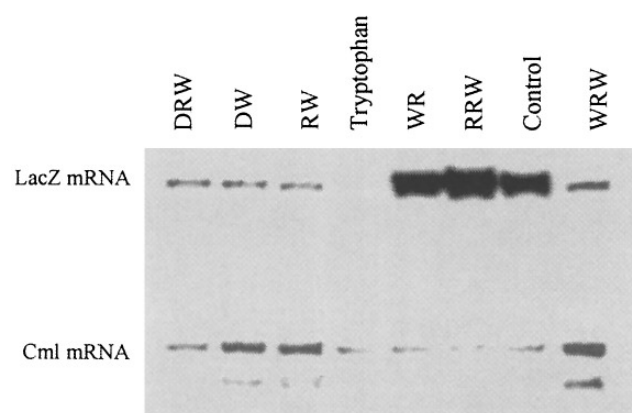

FIG. 2. Northern analysis of *LacZ* mRNA and the control *cml* mRNA transcripts as a result of peptide supplementation. Peptides were added in 100 μM concentrations. The *cml* gene is transcribed constitutively. The *LacZ* transcript is under control of the tryptophan operator. Distilled water was used as a control.

transcript. The addition of 100 μM RRW shows the highest level of transcription. WR and the control (distilled water) showed approximately the same level of transcription. The addition of 100 μM of peptides DW, DRW, WRW, RW and tryptophan showed little if any transcription. Care was taken to add same amount of RNA to each lane, nevertheless variations are seen in the control *cml* band indicating some discrepancy in total RNA from lane to lane. By normalizing the *cml* band the resultant levels of *lacZ* transcription are still in agreement with the previous β-galactosidase experiment (RRW $\gg$ WR, control $\gg$ WRW, DRW $>$ DW, RW, Tryptophan).

Gel Mobility Shift Analysis (GMSA)

The results from GMSA analysis using the various peptides, tryptophan and the control (distilled water) are shown in Figure 3. The addition of 1 mM of WRW, RW, DW, DRW or 0.5 mM of tryptophan to the incubation reaction containing the TrpR protein resulted in the formation of a TrpR-*trp OP* complex. 1 mM of peptides WR and RRW did not induce the formation of a TrpR-*trp OP* complex. The control did not induce a TrpR-*trp OP* complex. Figure 4 demonstrates a RRW dependent reduction in TrpR affinity for *trp OP* DNA. In the Figure 4 gel shift autoradiogram the protein concentration increases from right to left. Figure 4A shows the formation of TrpR protein and *trp OP* complexes in the absence of any peptide or corepressor, Figure 4B shows the formation of TrpR protein and *trp OP* complexes in the presence of 1 mM RRW. At TrpR protein extract concentrations of 25$\times$ and 10$\times$ comparison of the upper and lower gels reveals that the addition of 1 mM RRW inhibited the formation of TrpR DNA complexes.

Thin Layer Chromatography

Figure 5 shows two ninhydrin-stained thin layer chromatograms of gel mobility shift incubation reactions. The incubation mixture prior to chromatography was identical in all respect to the GMSA incubation reaction. The only difference between A and B in Figure 5 is that A lacks TrpR protein in the mixture. Scanning for spot intensity after background correction revealed no significant difference between the two chromatograms, A and B.

DISCUSSION

We have succeeded in modulating the TrpR protein using chemically synthesized di-and tri-peptides. The modulation of TrpR function was demonstrated both *in vivo* (β-galactosidase reporter system) and *in vitro* (GMSA). Previously we have modulated TrpR function through intragenic complementation between defective or weak TrpR monomer mutants (24, 25). A TrpR dimer

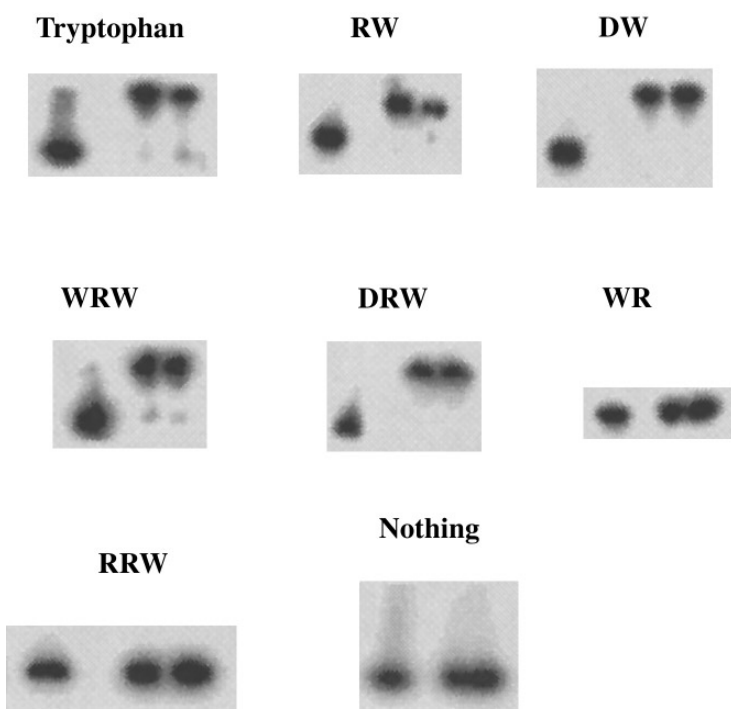

FIG. 3. Peptide induced formation of TrpR protein and Trp operator complexes. Gel mobility shift analysis of the 241 bp *trp EDCBA* operator region and partially purified TrpR protein in the presence of 1 mM peptide. Distilled water was used as a control.

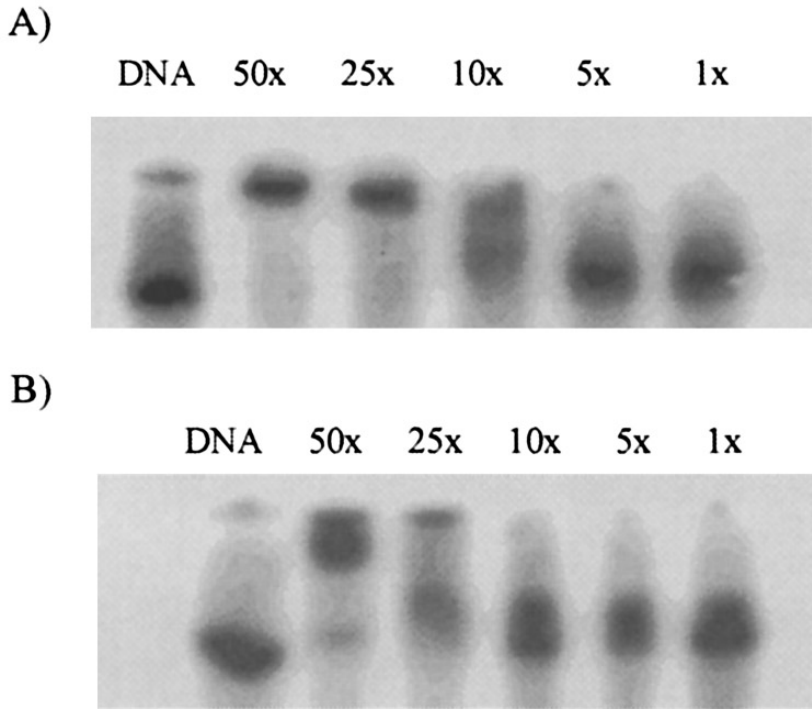

FIG. 4. The addition of RRW reduces the affinity of the TrpR protein for the 241 basepair Trp operator sequence. Gel mobility shift analysis on a 241 bp DNA fragment containing the *trp EDCBA* operator region and partially purified TrpR protein. Protein concentrations decrease from left to right. Figure A represents gel mobility shift without 1 mM RRW. Figure B represents gel mobility shift with 1 mM RRW in the incubation reaction buffer. Tryptophan was not present in the incubation reaction.

with T44M mutations on both monomers is unable to bind to the tryptophan operator region unless one monomer is complemented by a G85 (L,W, R, or K) mutant monomer to create a functional heterodimer (24, 25). In the present work we attempted to modulate the wild type repressor by adding small engineered peptides. Changes in the levels of transcription of the β-galactosidase reporter system are taken as an indication of changes in TrpR conformation. The TrpR corepressor pocket along with the corepressor tryptophan form the TrpR DNA binding face. The inclusion of the tryptophan residue in the engineered peptides was intended to target the peptides to the TrpR corepressor pocket. However, without a structural study we can not discern if the peptides were actually bound to the corepressor pocket.

Peptides possessing tryptophan at the carboxyl terminus were able to bind the tryptophan operator *in vivo* and *in vitro*. Peptides RW, WRW, DW, DRW repressed β-galactosidase production (Figure 1), as a result of small amounts of *LacZ* mRNA transcripts (Figure 2). This effect was due to the formation of TrpR-*trp OP* complexes as revealed by gel shift analysis (Figure 3). The peptide RRW produced large amounts of β-galactosidase (Figure 1) and *LacZ* mRNA transcripts (Figure 2), and also impeded the formation of a TrpR-*trp OP* complex in gel shift analysis (Figure 4). In contrast, the peptide WR containing a tryptophan residue at the amino terminus showed no ability to modulate the tryptophan operator. WR exhibit no significant effect on β-galactosidase production, transcribed similar amounts of *LacZ* transcript as the control (distilled water), and failed to induce TrpR-*trp OP* complex formation in gel shift analysis. The cloning vector pACYC184 without the TrpR coding sequence could not be differentially regulated by the presence of RRW, tryptophan, or water, indicating that the peptide modulators are acting specifically at the TrpR protein and not on another site within the plasmid (results not show).

Even in the absence of added tryptophan using the presented β-gal/reporter system the operator remains 90% repressed compared to the situation that arises in the complete absence of a functional TrpR protein (25). This could reflect constitutive transcription of the uncoupled tryptophan operon within the $\lambda R^{-}TLF$ strain (32) or TrpR protein binding to the sequence in the absence of tryptophan *in vitro* as can be seen in Figure 4. TrpR binding the operator in the absence of the corepressor tryptophan is likely to be an integral part of TrpR regulation.

The peptide RRW may bind to TrpR in such a way

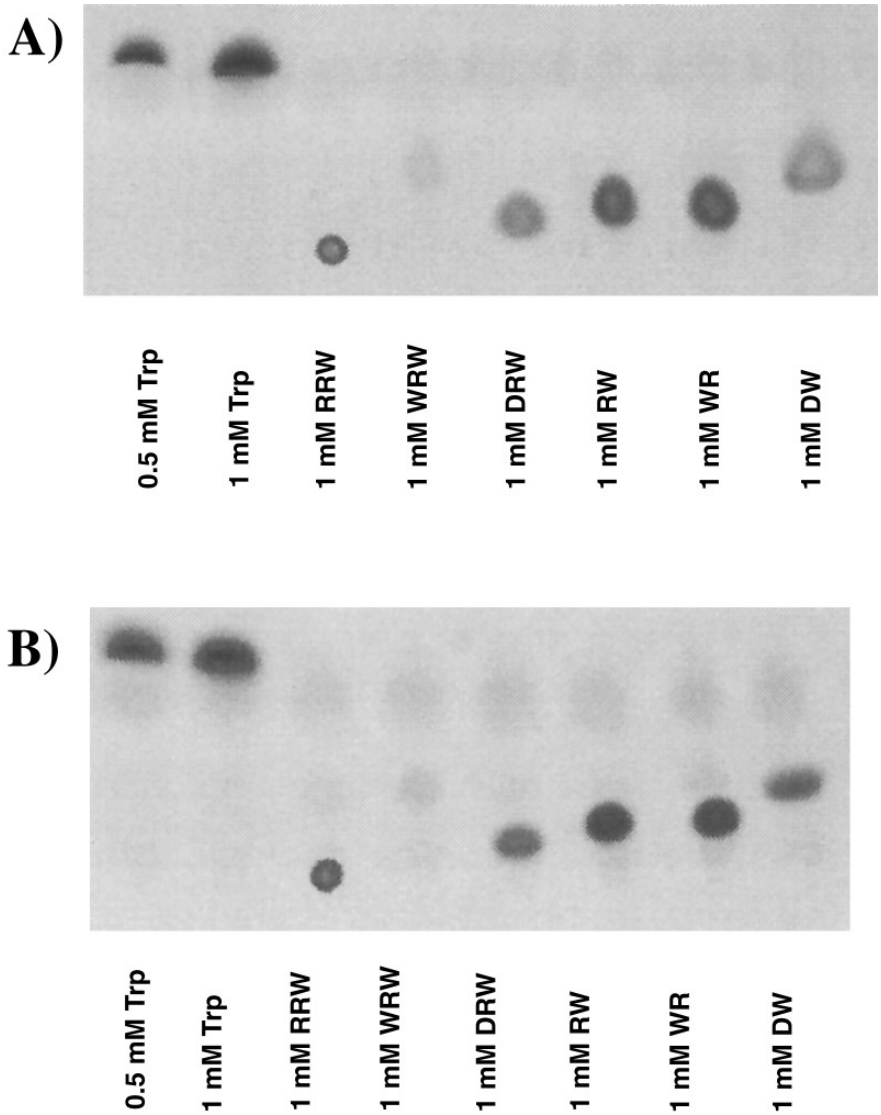

FIG. 5. Thin layer chromatography of ninhydrin stained gel retardation incubation reactions contaning indicated peptide. A) shows the incubation reaction without the TrpR protein extract present. B) shows the incubation reaction with the protein present. Densitometry scanning with background correction revealed no significant proteolysis of the peptides used due to the presence of the TrpR protein extract.

as to hamper non-specific binding to the operator thus acting as a de-repressor. This would lead to elevated levels of β-galactosidase transcription as seen in Figure 2. It would also explain how RRW manages to inhibit the binding of TrpR to the operator in the absence of tryptophan as seen in Figure 4. Indole acrylic acid (IAA) and indole propionate are well characterized inducers of TrpR (10, 18, 33). Indole propionate, a tryptophan analog lacking an amino group binds with high affinity to the TrpR aporepressor, however the resultant holorepressor has no affinity for the operator region and therefore can not repress transcription. Crystal structure analysis of the indole propionate (10) bound to TrpR show the bound indole propionate in the reverse oreintation of tryptophan such that the carboxyl group of indole propionate collides sterically and electrostatically with an operator phosphate thereby disrupting binding. Therefore the tryptophan amino group must play an important role in the orientation of tryptophan in the corepressor pocket. Peptides with a tryptophan carboxyl residue have affinity for the TrpR protein molecule but perhaps the nature of binding is decided by the remaining residues. Without a crystal structure we can not determine where the peptides bind the TrpR protein.

Thin layer chromatography showed no significant proteolysis of the peptides following the *in vitro* gel retardation incubation reaction. (Fig 5). In addition,

the fact that WR, RW, WRW, and RRW vary greatly in their ability to modulate the *trp OP* sequence *in vivo* makes it highly unlikely that what we are observing is simply the degradation of the peptides and the consequent release of free tryptophan into the media.

Active small peptide transport systems are well documented in bacteria (21), mammals (34) , fungi (35), and higher plants (36). Tryptophan containing peptides have been shown to bind with selectivity to guanine bases via aromatic stacking and hydrogen bond interactions (37–40). It is possible that the peptides are binding to the operator DNA sequence and not to the TrpR protein in such a way as to induce or inhibit binding to the operator. Both the classical CTAG operator halfsite (41–43) and the GNATC operator halfsite proposed by Yang *et al* (44) contain a guanine base. It should be noted that one would expect to see a difference in electrophoretic migration between a DNA fragment and a RRW bound DNA fragment. This was not the case in our studies. The peptides would also have to distinguish the TrpR consensus halfsites from the 4 $\times$ 10^6 base pairs of the *E. coli* genome.

In conclusion we have demonstrated both with *in vivo* and *in vitro* studies that synthetic peptides can modulate TrpR activity over a wide range of activity. This activity was reflected in β-galactosidase and northern analysis indicating direct transcriptional regulation of the reporter gene. This type of modulation of the TrpR protein is novel and represents a modulation strategy that does not include the alteration of protein primary structure. The implications of this strategy opens a wide range of potential applications within molecular biology and biotechnology.

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