Nucleic Acids Research, Vol. 20, No. 2 337–341

The NH_2-terminal arms of *trp* repressor participate in repressor/operator association

Barry K.Hurlburt+ and Charles Yanofsky*
Department of Biological Sciences, Stanford University, Stanford, CA 95403-5020, USA

Received August 19, 1991; Revised and Accepted December 11, 1991

ABSTRACT

The 3-dimensional structures of the *trp* repressor, aporepressor, and repressor/operator complex have been described. The NH_2-terminal arms of the protein, comprising approximately 12–14 residues, were not well resolved in any of these structures. Previous studies by Carey showed that the arms are required for full *in vitro* repressor activity. To examine the roles of the arms more fully we have removed codons 2–5 and 2–8 of the *trpR* gene and analyzed the resulting truncated repressors *in vivo* and *in vitro*. The Δ2–5 *trp* repressor was found to be approximately 25% as active as the wild type repressor *in vivo*. In *in vitro* equilibrium binding experiments, the Δ2–5 *trp* repressor was shown to be five-fold less active in operator binding. The rate of dissociation of the complex formed between the Δ2–5 *trp* repressor and operator was essentially the same as the rate of dissociation of the wild type *trp* repressor/operator complex. However association of the Δ2–5 *trp* repressor with operator was clearly defective. Since the NH_2-terminal arms of the *trp* repressor appear to affect association predominantly they may play a role in facilitating non-specific association of repressor with DNA as repressor seeks its cognate operators. The Δ2–8 *trp* repressor was unstable *in vivo* and *in vitro*, suggesting that some portion of the NH_2-terminal arm is required for proper folding of the remainder of the molecule.

INTRODUCTION

The *trp* repressor of *Escherichia coli* negatively regulates transcription initiation in the *trp*, *aroH*, *trpR* and *mtr* operons (1–5). Repression is believed to result from competition between repressor and RNA polymerase for overlapping sites in the respective promoter/operator regions (1–3, 6). The active form of the *trp* repressor is a symmetrical dimer consisting of interlocked polypeptides, and is complexed with two molecules of *L*-tryptophan (7, 8).

The *trp* aporepressor, a dimer of a 107 residue polypeptide, is organized into three types of structural domains (reviewed in ref. 9): symmetrically disposed identical 'reading heads', a central core, and identical amino terminal arms. Each reading head consists of a 'helix-turn-helix' DNA-binding motif (8). Activation of aporepressor by *L*-tryptophan binding displaces the mobile helix-turn-helix regions to positions appropriate for operator binding (10). The operators within the *trp*, *aroH* and *trpR* promoters bind repressor with near equal affinity, although they are located at different positions relative to the promoter elements (6). The *trp* operator contains a principle high-affinity binding site for *trp* repressor (11–13). Dissociation of the *trp* repressor/operator complex *in vivo* is rapid; a half life of three minutes was observed at 25°C (14). The operator base pairs most important for repressor recognition and binding *in vivo* are believed to be located in consecutive major grooves of the operator, and are well positioned for interaction with the symmetrically placed helix-turn-helix domains of the dimeric repressor (15–17). The 3-dimensional structure of a crystalline complex of the *trp* repressor and a symmetrical operator fragment revealed close association of segments of the protein and DNA (17). Unexpectedly, many of the contacts between *trp* repressor and the operator fragment occurred primarily through water-mediated hydrogen bonds. Based on this observation, a novel mode of nucleotide sequence recognition was proposed, termed 'indirect readout', in which the operator is recognized partly by its conformation rather than by hydrogen bonding of specific base pairs to the repressor (17). This model is controversial (18, 19).

The NH_2-terminal arms of *trp* repressor comprise the first 12–14 amino acid residues of each polypeptide (8). The structure of the arm was not resolved in any of the crystal structures (8, 10, 17). Chymotryptic removal of a peptide containing the amino terminal six residues (AlaGlnGlnSerProTyr) of the mature repressor polypeptide produced a dimeric repressor with reduced DNA-binding activity *in vitro* (12). In an effort to determine the function of the NH_2-terminal arm we have deleted codons 2–5 and 2–8 of the repressor structural gene, *trpR* and have attempted to express, purify and analyze the corresponding polypeptides. In this report we present the results of analyses of these deletion polypeptides. Deleting residues 2–5 of *trp* repressor reduced its repressing activity *in vivo* four fold. Using purified Δ2–5 *trp* repressor and quantitative filter binding, we demonstrated that operator binding was approximately five-fold reduced relative to that of wild type *trp* repressor. In kinetic analyses of association

* To whom correspondence should be addressed
+ Present address: Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

and dissociation of repressor with operator, we showed that the Δ2−5 *trp* repressor was defective only in association. Since the NH_2-terminal arms of *trp* repressor increase the rate of operator association one of their roles may be to facilitate repressor diffusion along the *E. coli* chromosome.

The Δ2−8 *trp* repressor appeared to be unstable in *E. coli* and in an *in vitro* transcription/translation system. A segment of the NH_2-terminal arms may be required for proper folding of the nascent polypeptide chain or for protection of the polypeptide from degradation.

MATERIALS AND METHODS

Bacterial strains and manipulations

E. coli strain TG1 (K12 Δ(*lac-pro*) *supE thi hsdD5/F' traD26 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15) was used as a general host for plasmid DNA replication. CY15082 (W3110 *tnaA2 lacU169* ΔtrpR-thr (λTLF1) was used for determination of *in vivo* repressor activity. λTLF1 contains the *trp* promoter/operator controlling a *trpL'-'lacZ* translational fusion (20). Following mutagenesis plasmid DNAs were transformed into DH5α cells (BRL, Bethesda, MD) and were purified from cultures of transformants by equilibrium centrifugation in CsCl gradients. Electroporation (BTX, San Diego, CA) was used to introduce recombinant plasmids into *E. coli* strains.

Mutagenesis and plasmid construction

To delete codons corresponding to residues in the NH_2-terminal arm, we introduced two new restriction sites into *trpR* by oligo-directed mutagenesis. The 110 bp *Bam*HI-*Sal*I fragment from pJPR2 (21) was cloned into pUC119 digested with *Bam*HI and *Sal*I (22). Single-stranded DNA from this plasmid (pBKH22) was used as template for oligo-directed mutagenesis using the thiol-nucleotide method (Amersham Corp., Arlington Heights, IL). An *Nco*I site overlapping codon 1 and an *Eag*I site overlapping codon 9 were introduced (pBKH23). The base changes did not alter the encoded amino acid residues. To delete codons 2−5 of *trpR* gene, a cassette of paired complementary oligomers was ligated into *Nco*I/*Eag*I cut pBKH23 yielding pBKH24. The small fragments from *Sma*I/*Sal*I digested pBKH23 and pBKH24 were ligated into *Eco*RV/*Sal*I digested pBKH13 creating pBKH20 (wild type) and pBKH28 (Δ2−5). pBKH13 was constructed by digesting pBR322 with *Nar*I, treating it with the large fragment of *E. coli* DNA polymerase I in the presence of the four nucleoside triphosphates, and ligation (pBRNN). The 438 bp *Bam*HI fragment containing the *trpR* gene from pRLK13 (20) was inserted into the *Bam*HI site of pBRNN to generate pBKH13.

To delete codons 2−8 the small *Bam*HI fragment from pJPR2 (21) was cloned into the replicative form of M13mp8. Codons 2−8 were deleted using oligo-directed mutagenesis. The resulting gene was cloned into the *Bam*HI site of pACYC184 (pJPR4). The wild type *trpR* gene (*Bam*HI fragment) from pJPR2 cloned into the *Bam*HI site of pACYC184 (pJPR3) served as the wild type control for *in vivo* activity determinations. For all *in vivo* activity measurements *trp* repressor was expressed from the *tet* promoter.

For high-level expression of the wild type, Δ2−5 and Δ2−8 *trp* repressors, the small *Bam*HI fragments from the low copy plasmids pBKH20 (wild type), pBKH28 (Δ2−5) and pJPR4 (Δ2−8) were cloned into plasmid ptacterm (21). All constructions were verified by sequencing. The sequence of the NH_2-terminal arm is shown below. The Δ2−5 and Δ2−8 *trp* repressors were generated by deleting the nucleotides within parentheses.

	1	2	3	4	5	6	7	8	9	10	11	12
ACC	ATG	(GCC	CAA	CAA	TCA)	CCC	TAT	TCG)	GCC	GCG	ATG	GCA
	Met	Ala	Gln	Gln	Ser	Pro	Tyr	Ser	Ala	Ala	Met	Ala

Expression and purification of wild type and Δ2−5 *trp* repressors

Wild type and Δ2−5 *trp* repressors were produced and purified as described (21). The purity of repressor preparations was assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Repressor preparations were estimated to be approximately 95% pure by visual inspection. Repressor concentrations were determined spectrophotometrically using an extinction coefficient of 1.2 $cm^{-1}mg^{-1}ml$ at 280 nm (7). The amino terminal 10 residues of the purified Δ2−5 *trp* repressor were sequenced. Residues 1−10 were: ProTyrSerAlaAlaMet-AlaGluGlnArg. The amino terminal methionine of the Δ2−5 *trp* repressor had been removed posttranslationally as has been observed for the wild type *trp* repressor following high level expression (2, 7).

Assays for repressor activity *in vivo* and *in vitro*

In vivo repressor assays−Cells of strain CY15082 containing plasmids pBKH20, pBKH28, pBRNN, pJPR3, pJPR4 or pACYC184 were grown at 37°C to mid-log phase in minimal medium (23) plus 0.2% glucose, 0.2% acid hydrolyzed casein, 100 μg/ml *L*-threonine, either 100 μg/ml ampicillin (pBRNN and derivatives) or 20 μg/ml chloramphenicol (pACYC184 and derivatives), and 20 μg/ml tryptophan (when appropriate). β-galactosidase assays were performed as described by Miller (24) using chloroform and SDS to permeabilize cells.

In vitro repressor assays−Filter binding assays and preparation of the 43 bp ^{35}S-labelled operator were performed as described (14).

Determination of equilibrium and kinetic constants

The equilibrium dissociation constant (K_D), association rate constant (k_a) and dissociation rate constant (k_d) were calculated using the equations presented by Riggs and coworkers (25, 26).

RESULTS

Relative *in vivo* activity of wild type and Δ2−5 *trp* repressors

Repressor activity was examined *in vivo* by introducing appropriate plasmids into the single lysogen reporter gene strain CY15082. This strain contains the λTLF1 phage genome carrying a *trpL'-'lacZ* translational fusion driven by the *trp* promoter/operator. The chromosomal *trpR* gene was deleted from this strain. *trpR* was expressed constitutively at a low level from the *tet* promoter of the introduced plasmids. CY15082 cells containing pBRNN (vector), pBKH20 (wild type *trp* repressor) and pBKH28 (Δ2−5 *trp* repressor) grown in the presence and absence of tryptophan were examined. The wild type *trp* repressor (pBKH20) reduced λTLF1 expression to 5% in the absence of tryptophan and to 0.1% in its presence (Table 1). The Δ2−5 *trp* repressor showed the same differential response to tryptophan, 50-fold, but in the presence or absence of tryptophan the β-galactosidase levels were approximately four-fold higher than those for the wild type *trp* repressor. These findings indicate that the Δ2−5 *trp* repressor is approximately 25% as active as the wild type repressor *in vivo*.

Table I. *In vivo* β-Galactosidase Activity, wild type vs. Δ2−5 *trp* repressor

plasmid	*trp*R allele on plasmid	+Trp	−Trp	−Trp/+Trp
pBRNN	none	15,000	15,000	1
pBKH20	wild type	13(0.1)	700(5)	54
pBKH28	Δ2−5	55(0.3)	2,700(20)	49

Determination of wild type *trp* repressor and Δ2−5 *trp* repressor activities *in vivo*. *E. coli* strain CY15082 contains a single copy *trpL'-'lacZ* translational fusion driven by the *trp* promoter/operator. *trp* R genes were expressed constitutively from low copy plasmids. CY15082 cells containing the indicated plasmids were grown in media with or without *L*-tryptophan as described by Kelley and Yanofsky (20). β-galactosidase assays were performed according to the procedure of Miller and enzyme activity is presented in Miller units (24). The percentage of the control (pBRNN) value is shown in parentheses.The values presented are the averages of three experiments. Variation of activity values between experiments was less than 10%.

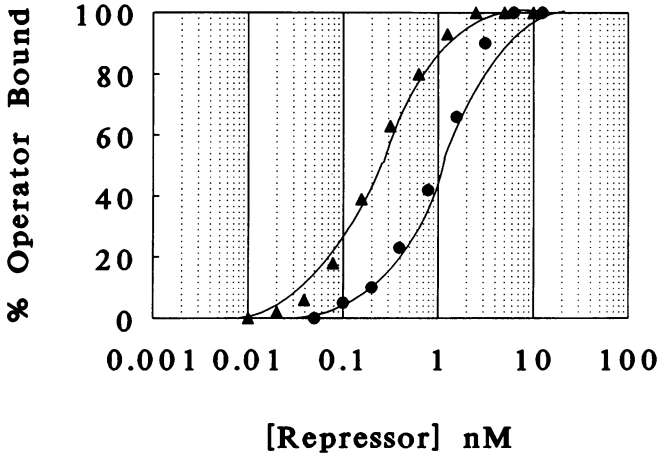

Figure 1. Determination of K_D values for the wild type (▲) and Δ2−5 *trp* repressors (●). Wild type *trp* repressor and Δ2−5 *trp* repressor were incubated with ^{35}S-labelled operator DNA (1×10^{-11}M) in filter binding buffer B (FBB, ref. 14) plus 300 μM tryptophan. The repressor concentration was varied from 0.01 to 10 nM. In filter binding experiments with *trp* repressor under our standard conditions less than 5% of the available operator was retained on filters in the absence of repressor. This background retention was subtracted. Saturation of operator occurred when approximately 60% of the operator was retained on filters following sample filtration and washing. This saturation value was taken as 100% binding and the data were normalized appropriately. The values presented are the averages of three experiments.

Equilibrium binding of wild type and Δ2−5 *trp* repressors in the presence of saturating tryptophan

To determine the biochemical basis of the reduced *in vivo* activity of the Δ2−5 *trp* repressor, we overexpressed and purified this repressor from *E. coli*, and examined its operator binding *in vitro*. The results of equilibrium binding experiments between operator and wild type or Δ2−5 *trp* repressors are presented in Figure 1. In these analyses a fixed, limiting concentration of ^{35}S-labelled operator (2×10^{-11} M) was incubated with increasing concentrations of repressor, the mixtures incubated, and then filtered. With the Δ2−5 *trp* repressor, a five-fold higher protein concentration was required to retain 50% of the available operators on filters. The apparent K_D (25) for the Δ2−5 *trp* repressor was determined to be 1 nM, while that for the wild type *trp* repressor under the conditions used was 0.2 nM. The higher observed K_D of the Δ2−5 *trp* repressor is sufficient to account for its reduced activity *in vivo*.

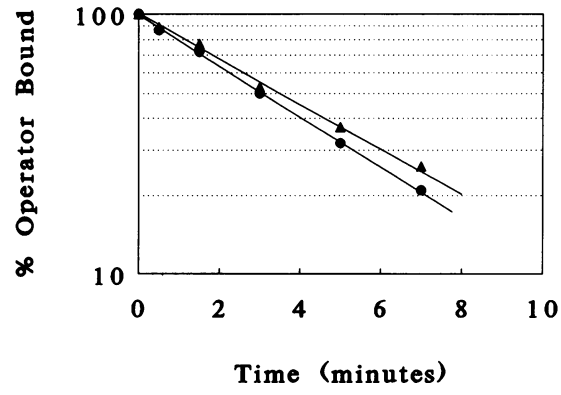

Figure 2. Determination of the half lives of repressor/operator complexes formed with wild type *trp* repressor (▲) or Δ2−5 *trp* repressor (●). Repressor protein (10 nM) and ^{35}S-labelled operator DNA (1 nM) were incubated in filter binding buffer B (FBB, ref. 14) containing 300 μM tryptophan. At time 0, a 500-fold excess of unlabelled operator was added. Samples were taken at the times indicated and filtered. The amount of radioactivity retained on the filter was determined and normalized to 100% at time 0. The values presented are the averages of three experiments.

Kinetics of dissociation of *trp* repressor/operator complexes

The weaker equilibrium binding by the Δ2−5 *trp* repressor could be the result of a reduced rate of complex formation, more rapid dissociation of the complex, or a combination of the two. To compare dissociation rates we determined the half lives of the complexes of wild type *trp* repressor and Δ2−5 *trp* repressor with operator (Fig. 2). Purified repressor and radiolabelled operator were equilibrated in the presence of excess tryptophan. At t = 0, a 500-fold molar excess of unlabelled operator was added. Samples were removed at the times indicated and filtered (Fig. 2). The Δ2−5 *trp* repressor/operator complex dissociated only slightly faster than the wild type *trp* repressor/operator complex. The half life of the wild type repressor/operator complex was 3.5 minutes under the conditions employed, while the half life of the Δ2−5 *trp* repressor/operator complex was 3 minutes. The difference in the rate of complex dissociation therefore cannot account for the five-fold decrease in equilibrium binding of the Δ2−5 *trp* repressor compared to the wild type repressor.

Kinetics of association of wild type *trp* repressor versus Δ2−5 *trp* repressor with operator

We used the filter binding assay to measure the rates of complex formation directly (Fig. 3). Solutions of purified repressor and operator were mixed at t = 0, and samples were removed at the times indicated and filtered. Excess tryptophan was present in both the protein and DNA solutions. Formation of the wild type *trp* repressor/operator complex proceeded very rapidly; saturation was achieved by 15−20 seconds, the time of first sampling. We could not obtain intermediate values for association of wild type *trp* repressor with operator. We attempted these measurements at lower repressor and operator concentrations, but the counting errors were too high to give reliable results. By contrast, approximately 60−70 seconds were required for operator saturation with the Δ2−5 *trp* repressor (Fig. 3). A K_a of $1.2\times10^{8}M^{-1}sec^{-1}$ was calculated for the Δ2−5 *trp*

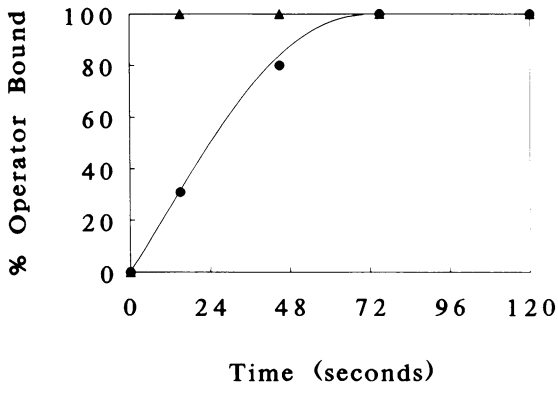

Figure 3. Determination of the rate of complex formation for wild type *trp* repressor (▲) or Δ2−5 *trp* repressor (●) with operator. Repressor protein (2×10^{-10} M) and ^{35}S-labelled operator (1×10^{-11} M) were mixed at t = 0. Both repressor and operator were in filter binding buffer B (FBB, ref. 14) plus 300 μM tryptophan. Samples were removed at various times and filtered. The amount of radioactivity retained was determined. Background retention was subtracted to reflect 0% binding at t = 0. 100% binding was the amount of operator retained on filters when the samples had reached equilibrium (t > 10 minutes). The values presented are the averages of three experiments.

repressor/operator interaction (26). The slower rate of complex formation for the Δ2−5 *trp* repressor is consistent with the higher K_D for wild type repressor and operator versus Δ2−5 *trp* repressor and operator. These results demonstrate that deleting residues 2−5 of *trp* repressor affects its ability to associate with operator. From the measured values of K_D and k_d we calculated k_a for the wild type *trp* repressor/operator interaction to be 1.2×10^9 M^{-1} sec^{-1}. This suggests that the association rate is too rapid to be measured with the filter binding assay under these conditions.

DISCUSSION

Crystallographic analyses with the *E. coli trp* repressor have failed to reveal the function of its NH_2-terminal arms. Proteolytic cleavage between residues 7 and 8 yielded a truncated repressor that was defective in operator binding in the gel mobility-shift assay (12). We analyzed the function of the NH_2-terminal arms by deleting codons 2−5 of the *trpR* gene and examining the properties of the resulting deletion repressor. We found that the truncated repressor was only about 25% as active as the wild type repressor *in vivo*. Equilibrium filter binding studies demonstrated that the Δ2−5 *trp* repressor was approximately five-fold less active than wild type repressor in operator binding *in vitro*, agreeing well with the four-fold reduction in *in vivo* activity that was detected. The half lives of complexes of the Δ2−5 *trp* repressor and the wild type *trp* repressor with operator DNA were essentially the same. However the calculated association rate constant for the wild type *trp* repressor was five-fold higher than that of the Δ2−5 *trp* repressor. Apparently removal of residues 2−5 of the NH_2-terminal arms of the repressor has a significant effect only on operator association.

The reduction in operator affinity and in *in vivo* repressing activity observed with the Δ2−5 *trp* repressor was considerably less than the fifty-fold reduction reported for proteolytically produced Δ2−7 *trp* repressor (12). The two additional residues removed or the conditions used in the mobility shift assay (pH 6.0) may have accentuated differences in binding affinities. The Δ2−7 *trp* repressor was reported to be only 2- to 3-fold less active than wild type repressor in a phosphatase protection assay (27).

In the course of this study we also introduced a deletion that should remove residues 2−8 of the *trp* repressor. The resulting Δ2−8 *trp* repressor was apparently unstable. We could find no conditions in which repressor activity or protein could be detected following expression of the Δ2−8 *trpR* gene *in vivo* or *in vitro*. Presumably the NH_2-terminal arms participate in an essential step in folding of *trp* repressor, or contribute to its stability.

On the basis of the results presented in this report we believe that the NH_2-terminal arms of *trp* repressor play a crucial role in DNA association. In contrast, amino acid changes in the central core region of the repressor increase the stability of the repressor/operator complex, but do not affect association (14). These findings suggest that one domain of the protein maintains the DNA complex once formed, while a second contributes to complex formation predominantly. Carey (12) observed no differences between the hydroxyl radical footprints of wild type *trp* repressor and the Δ2−7 *trp* repressor on operator and concluded that the NH_2-terminal arms do not make unique contacts when the repressor is complexed with operator. In agreement with this conclusion, as mentioned, the NH_2-terminal arms were unresolved in the crystal structure of *trp* repressor/operator complex (17). The arms of the *trp* repressor do not appear to serve the same role as the arms of λ repressor which contribute to maintaining the protein/DNA complex (28, 29).

We can suggest two possible roles for the NH_2-terminal arm domain of *trp* repressor. The NH_2-terminal arms may facilitate repressor's scanning of chromosomal DNA in its search for its cognate operators. Facilitated diffusion, diffusion at rates faster than random diffusion, has been demonstrated for *lac* repressor (30) and *cro* repressor (31) and is thought to proceed by low affinity, electrostatic interactions between residues in these repressors and DNA. Facilitated diffusion presumably reduces the volume of the cell within which a regulatory protein must search for its target operator (reviewed in ref. 32). The association rate constant (k_a) for the interaction of the wild type *trp* repressor with the 43 bp operator was calculated from K_D and k_d to be 1.2×10^9 $M^{-1}sec^{-1}$. The k_a for the Δ2−5 *trp* repressor calculated from the results of the association rate experiments is 1.2×10^8 M^{-1} sec^{-1} and from calculations based on K_D and k_d is 2×10^8 $M^{-1}sec^{-1}$. The theoretical association rate constant based on 3-dimensional diffusion is approximately 1×10^8 $M^{-1}sec^{-1}$ (32). Thus, the k_a for the Δ2−5 *trp* repressor is very similar to that postulated for 3-dimensional diffusion, while that for the wild type *trp* repressor is somewhat more rapid. The k_a for the *cro* repressor and operator interaction varied from 3×10^8 $M^{-1}sec^{-1}$ with a 20 bp operator to 4.5×10^9 $M^{-1}sec^{-1}$ with the largest operator containing DNAs tested (31). Based on our results, it is likely that the NH_2-terminal arms of the *trp* repressor mediate facilitated diffusion, even though the 43 bp operator DNA is not a particularly large target for nonspecific binding. Residues 3(Gln), 4(Gln), and 5 (Ser) of the NH_2-terminal arm are potential hydrogen bond partners. Based on this hypothesis, the Δ2−5 *trp* repressor should have reduced affinity for non-operator DNA compared to the wild type *trp* repressor. We were unable to measure non-operator binding accurately using the filter binding assay. Further studies will explore the role of facilitated diffusion in *trp* repressor action, and will examine the possible involvement of repressor's NH_2-terminal arms in such a process.

In conjunction with facilitated diffusion, the NH_2-terminal arms of *trp* repressor could enhance the rate of complex association by orienting the binding surface of the repressor towards the DNA. In effect, this would increase the productivity of 'collisions' between repressor and DNA to only those involving the binding surface of the protein. Taken together, these two potential functions of the NH_2-terminal arms could result in the enhanced rate of complex formation for the wild type *trp* repressor compared to the Δ2−5 *trp* repressor.

ACKNOWLEDGEMENTS

We thank Janet L. Paluh for construction of the Δ2−8 *trpR* gene and Virginia Horn for initial *in vivo* analysis of the Δ2−8 *trp* repressor. We also thank Jannette Carey, Charlotte Peterson and Paul Sigler for critical reading of the manuscript. These studies were supported by National Science Foundation Grant DMB-8703685. B.K.H. was supported by National Institutes of Health-National Research Service Award GM11980. C.Y. is a career investigator of the American Heart Association.

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