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# Formation of Heterodimers Between Wild Type and Mutant *trp* Aporepressor Polypeptides of *Escherichia coli*

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***ABSTRACT*** **Availability of the three-dimensional structure of the *trp* repressor of *Escherichia coli* and a large group of repressor mutants has permitted the identification and analysis of mutants with substitutions of the amino acid residues that form the tryptophan binding pocket. Mutant aporepressors selected for study were overproduced using a multicopy expression plasmid. Equilibrium dialysis with $^{14}$C-tryptophan and purified mutant and wild type aporepressors was employed to determine tryptophan binding constants. The results obtained indicate that replacement of threonine 44 by methionine (TM44) or arginine 84 by histidine (RH84) lowers the affinity for tryptophan approximately two- and four-fold, respectively. Replacement of arginine 54 by histidine (RH84) or glycine 85 by arginine (GR85) results in complete loss of tryptophan binding activity. Purified mutant and wild type aporepressors were used in in vitro heterodimer studies. The *trp* repressor of *E. coli* functions as a stable dimer. A large number of *trp* repressor mutants produces defective repressors that are transdominant to the wild type repressor in vivo. The transdominance presumably results from the formation of inactive or slightly active heterodimers between the mutant and wild type polypeptide subunits. An in vitro assay was developed to detect and measure heterodimer formation. Heterodimer formation was thermally induced, and heterodimers were separated on nondenaturing polyacrylamide gels. Aporepressors readily formed heterodimers upon treatment at 65°C for 3 minutes. Heterodimer formation was significantly retarded by the presence of the corepressor, L-tryptophan. Indole-3-propionic acid, 5-methyl tryptophan, and other analogs of tryptophan, as well as indole, also inhibited heterodimer formation. These results indicate that the presence of the indole moiety in the corepressor binding pocket increases the stability of the dimer.**

**Key words: DNA binding protein, ligand binding, equilibrium dialysis, dimer, stability**

## INTRODUCTION

The *trp* repressor of *Escherichia coli* regulates expression of genes required for the de novo biosynthesis of tryptophan in response to changes in the intracellular concentration of its corepressor, L-tryptophan.[1–6] The *trp* aporepressor is activated by the binding of two molecules of its corepressor. Once activated, *trp* repressor binds to the operators of the *trp, aroH,* and *trpR* operons, regulating transcription initiation.[7,8] The *aroH* and *trp* operons encode biosynthetic enzymes, whereas the *trpR* operon encodes the *trp* aporepressor. The *trpR* gene has been cloned and its nucleotide sequence determined.[3] The aporepressor and repressor have been purified and the crystal structures of both have been solved at high resolution.[9–11] The *trp* aporepressor is a dimer of identical 107 residue polypeptides.[11] The existence of the three-dimensional structures, and the availability of many repressor mutants,[2,12] has facilitated this analysis of the in vivo and in vitro tryptophan-binding activity of mutant repressors with changes of those amino acid residues predicted to participate in tryptophan binding.

A class of *trpR* mutants was identified that have a transdominant effect when present in a strain bearing a chromosomal copy of the wild type repressor gene.[12] The transdominant phenotype ($trpR^{-d}$) is thought to result from the formation of heterodimers defective in repressor activity.[13] Analogous results have been observed with mutant forms of other repressors, such as the *lac* repressor.[14,15] The chain terminating *trpR* nonsense mutant Qam68 produces a truncated polypeptide lacking the C-terminal 40-amino acid residues that contain the polypeptide segment involved in DNA recognition. Since the Qam68 mutant displays strong transdominance, it is likely that the first 67 residues of the *trpR* polypeptide are sufficient for negative complementation.

The majority of the $trpR^{-d}$ missense mutations (71%) map to a gene segment corresponding to the D-helix-turn-E-helix region of the repressor, which is believed to interact directly with operator DNA. A second group of transdominant missense mutations results in the substitution of residues that form the surface of the corepressor binding pocket.[9,11]

In the present study we have purified four mutant *trp* aporepressors that have substitutions of residues believed to comprise the corepressor binding site. Us-

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ing equilibrium dialysis analyses, we showed that these mutant aporepressors are partially or totally defective in L-tryptophan binding. We also developed an in vitro procedure for generating heterodimers from purified wild type and mutant *trp* aporepressors. The net charge differences between wild type aporepressor and some mutant species, and their heterodimers, allowed us to separate heterodimers electrophoretically on nondenaturing polyacrylamide gels, and to quantitate their formation. We have demonstrated that L-tryptophan and certain tryptophan analogs inhibit heterodimer formation.

## MATERIALS AND METHODS

### Chemicals Strains Plasmids, and Media

L-tryptophan, D-tryptophan, L-phenylalanine, D,L-tryptophan, D,L-5-methyltryptophan, tryptamine, indole, and indole-3-propionic acid were purchased from Sigma. Overexpressing plasmids were constructed by incorporating *Bam*HI fragments containing the various mutant *trpR* alleles into expression plasmid ptacterm, as described.[16] In this plasmid, the *tac* promoter drives *trpR* mRNA production. The recipient strain used for overproduction of the *trp* aporepressor was CY15071 (W31 10 *tnaA2*Δ*trpR thr lacI*$^{Q}$). This strain has *trpR* deleted and carries *lacI*$^{Q}$ for control of the *tac* promoter of the expression plasmid. Each expression plasmid was introduced into CY15071, and *trp* aporepressor synthesis was induced by addition of IPTG, as described.[16]

Repression was measured in vivo using a lysogenic recipient, strain CY15058 (W3110 *tnaA2 trpR thr*-Δ*lac*U169/λTLF1), in which the single integrated copy of the lambda genome contains a *trp* promoter/operator *trpL'-'lacZ* gene fusion. Production of hybrid β-galactosidase by this strain is controlled by repression of transcription initiation at the *trp* promoter of λTLF1. To measure in vivo repression by any of the mutant repressors, derivatives of pRLK13[12] containing the various mutant alleles were introduced into CY15058, and bacterial cultures were grown in minimal medium[17] containing 0.2% glucose, 0.2% casein hydrolysate, with or without 40 μg/ml tryptophan, as indicated. Culture media generally contained either 30 μg/ml chloramphenicol or 100 μg/ml ampicillin to stabilize the appropriate plasmid. Plasmid pRLK13 is a derivative of pACYC184 in which *trpR* expression is driven by the *tet* promoter.[12]

### β-Galactosidase Assays

Strain CY15058 containing plasmids pRLK13, pTM44, pRH54, pRH84, or pGR85 was grown at 31°C (the λTLF1 prophage is a λcI857 derivative) in the media described above and β-galactosidase assays were performed as described by Miller.[18]

### Purification of Mutant *trp* Aporepressors

Mutant aporepressors were overproduced in *E. coli* and purified as previously described.[16] Comparable yields were obtained with each repressor, indicating that none was particularly labile. Dialyzed aporepressor preparations were concentrated using an Amicon ultrafiltration apparatus and P10 membranes. Protein concentration was determined spectrophotometrically.[19] The repressor was stored frozen at −20°C in buffer containing 0.2M NaCl at a protein concentration of 4–7 mg/ml.

### Equilibrium Dialysis

The affinity of L-tryptophan for pure wild type and mutant *trp* aporepressors was determined using equilibrium dialysis measurements with L-[methylene-$^{14}$C] tryptophan (sp. act. 53.5 mCi/mmol, Amersham). An eight-place Hoeffer equilibrium microdialyzer module with EMD104 membranes (6,000–8,000 dalton cutoff), driven by a rotary motor, was used in this study. Each chamber had a capacity of 100 μl on each side of the dialysis membrane. The dialysis solution contained 10 mM Tris-HCl, pH 7.8, and 0.1 M NaCl. Aporepressor at a final concentration of 40 μM was placed on one side of the chamber, and various concentrations of L-[methylene-$^{14}$C] tryptophan were placed on the other side. Dialysis was carried out overnight (16–18 hours) at 4°C. All chambers were emptied, and 10 μl aliquots of each were counted in a scintillation counter.

### Samples for Heterodimer Formation

Standard conditions for formation of heterodimers were as follows: two purified *trp* aporepressors, at the same concentration, were combined, so that 0.15 nmoles of total dimeric protein (3.75 μg protein) were present in a final volume of 3–6 μl (25–50 μM) in 100 mM Tris buffer (pH 6.8), 50–100 mM NaCl. Where indicated, L-tryptophan was added. Samples were then heated in water baths at various temperatures for various times. After heating, samples were cooled rapidly in an ice bath. Three microliters of 80% glycerol with 0.0025% marking dye were added to each sample before loading the entire sample on a gel. The rate of heterodimer formation was not affected by changing the pH of the Tris buffer from 6.8 to 8.8. Samples to be examined for heterodimer formation induced by ethanol were prepared essentially as above, except that tryptophan was added prior to the ethanol, and samples were incubated at either 22°C or 37°C for 15 minutes.

### Nondenaturing Polyacrylamide Gel Electrophoresis

Both alkaline continuous and neutral discontinuous native polyacrylamide gel buffer systems were used.[20,21] Neither of these buffer systems induced heterodimer formation. On the other hand, alkaline discontinuous and neutral continuous electrophoretic conditions gave results that were not always reproducible (data not shown).

### Measurement of Heterodimer Formation by Densitometry

Coomassie stained gels were scanned with a Biomed Instruments Soft Laser Scanning Densitometer. The data were plotted and integrated using a Biomed Instruments Videophoresis Analysis Program in conjunction with an Apple IIe computer system. The inset in Figure 1C shows that the fraction of total protein determined spectrophotometrically[19] agreed with the fraction of the total signal obtained from a densitometry trace of stained bands produced from serially diluted protein. The linearity of signal response permitted the fraction of total protein per sample to be determined. The fractional abundance of homodimers and heterodimers per sample was calculated by scanning the total signal from each sample.

## RESULTS

### In Vivo Activity of Mutant *trp* Repressors

The in vivo activities of four mutant *trp* repressors, constitutively expressed from the *tet* promoter of pACYC184, were determined using cultures growing in medium with and without tryptophan supplementation. It can be seen in Table I that the four mutant repressors have decreased activity in vivo, in the presence or absence of exogenous tryptophan. Two repressors, TM44 and RH84, showed a slight tryptophan response in vivo, while GR85, which was slightly active, was unaffected by the presence of the tryptophan supplement. Preliminary in vitro filter binding experiments indicate that RH54 repressor does not bind operator DNA within the limits of detection (L.S. Klig, unpublished results). We would not expect any of these mutant repressors to bind operator DNA in vitro.

### Tryptopan Binding by Mutant and Wild-Type *trp* Repressors

The four mutant aporepressors were overproduced to the same extent in *E. coli,* suggesting that they are equally stable. They also behaved similarly to the wild type repressor during purification. The electophoretic mobility of the mutant polypeptides during SDS-PAGE was indistinguishable from that of the wild type polypeptide, indicating that they are normal length. The electrophoretic mobilities of the mutant aporepressors in nondenaturing gels were consistent with their being stable dimers, with the expected charge differences (Figs. 1 and 2). Formation of heterodimers with intermediate mobilities (see below) supports this interpretation. Negative complementation analyses in vivo[12] also indicate that mutant aporepressor polypeptides can readily form heterodimers with the wild type polypeptide. Taken together, these findings suggest that the dimeric mutant aporepressors are structurally similar to the wild type protein.

The binding of L-tryptophan by pure wild type and mutant repressors was measured using equilibrium dialysis (see Materials and Methods). Wild type, TM44, and RH84 *trp* aporepressors bind two molecules of tryptophan in a noncooperative manner. The $K_S$ value of $23 \pm 5$ $\mu$M for wild type repressor is essentially indistinguishable from the previously reported binding constants measured at 4°C.[22,23] The $K_S$ values obtained with the four mutant aporepressors are listed in Table I. All four are defective in tryptophan binding; aporepressors RH54 and GR85 are devoid of tryptophan binding activity in the presence of 1 mM L-tryptophan, while the TM44 and RH84 aporepressors have only slightly reduced binding activity. The TM44 and RH84 repressors respond

**TABLE I. The Amino Acid Replacements in *trpR* Mutants Presumed Altered in the L-Tryptophan Binding Site, Tryptophan Binding, and In Vivo Repressor Activity***

| Amino acid Wild type (mutant) | Position | *trpR* Allele on plasmid | In vivo β-gal activity −Trp | In vivo β-gal activity +Trp | $K_S$ L-tryptophan ($\mu$M) |
|---|---|---|---|---|---|
| | | None | 12,000 | 12,000 | — |
| | | Wild type | 500 | 10 | $23 \pm 5$ |
| Threonine (methionine) | 44 | TM44 | 9,000 | 5,900 | $39 \pm 7$ |
| Arginine (histidine) | 54 | RH54 | 11,000 | 11,200 | N.B. |
| Arginine (histidine) | 84 | RH84 | 10,100 | 5,300 | $88 \pm 4$ |
| Glycine (arginine) | 85 | GR85 | 2,100 | 2,000 | N.B. |

*Wild type and mutant plasmids were introduced into a $trpR^-$ strain containing a repression indicator (λ *trp* promoter/operator-*trpL'-' lacZ* fusion), and β-galactosidase (β-gal) activities were determined using cultures grown with and without 20 μg/ml L-tryptophan. The $K_S$ values for tryptophan binding, obtained from duplicate equilibrium dialysis measurements with pure aporepressors, are presented in the last column. N.B. stands for no detectable binding up to a concentration of 1 mM L-tryptophan.

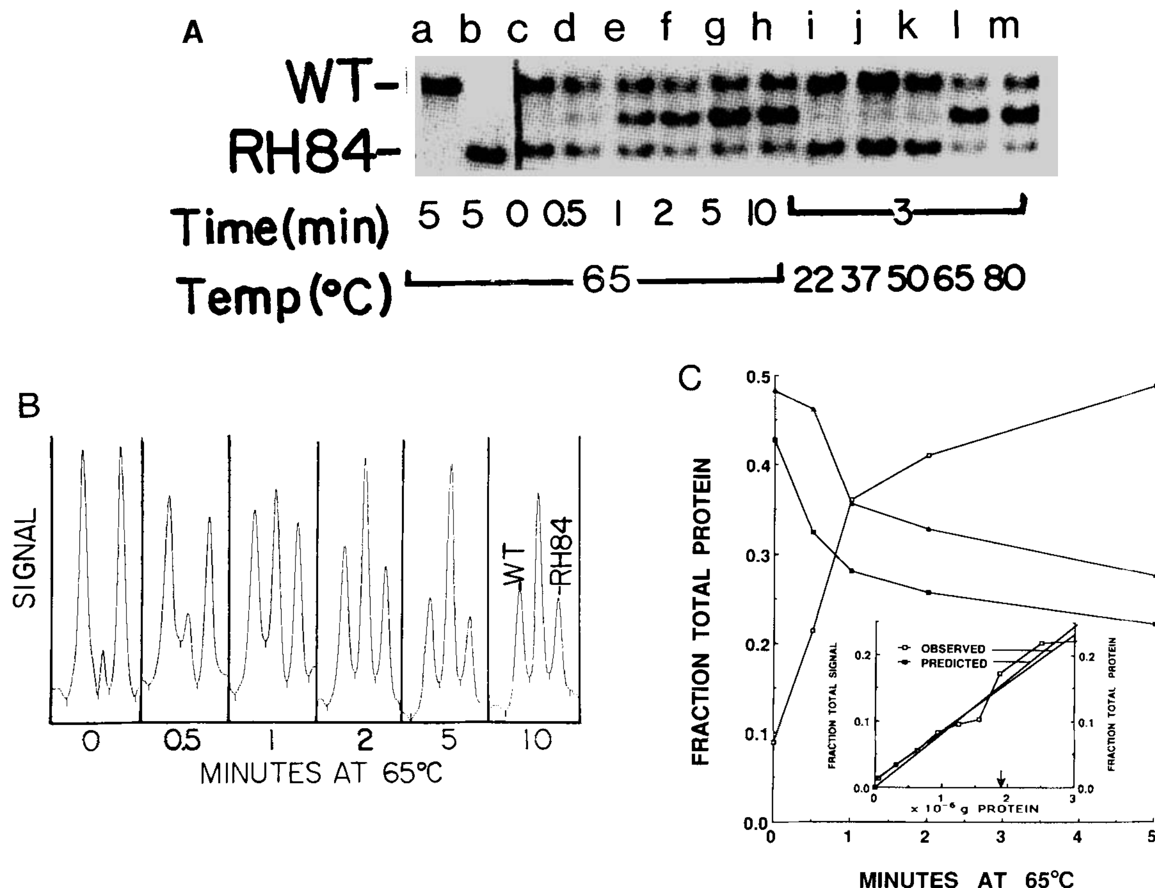


Fig. 1. **A:** Native alkaline continuous polyacrylamide gel stained with Coomassie brilliant blue R. Samples of wild-type (WT) and mutant (RH84) aporepressors were treated at times and temperatures indicated. **B:** Densitometer traces used to obtain this plot. **C:** Plot of percent total protein from the integrated values obtained from a densitometer trace: □, WT/RH84 heterodimer; ▲, WT homodimer; ■, RH84 homodimer. Inset shows a plot of the data obtained from a densitometer trace of a serial dilution of RH84 protein (concentration determined spectrophotometrically) against the fraction of total signal (□). A least squares calculation of these data yielded the line marked "observed." The fraction of total protein is denoted by the line marked "predicted." It is the amount of protein per sample in the serial dilution (determined spectrophotometrically) divided by the total amount of protein in the combined samples. The arrow denotes one-half the total protein concentration per sample.

slightly to tryptophan in vivo, in agreement with the results of these in vitro binding studies.

### Heterodimer Formation

Homodimers of purified mutant and wild type (WT) aporepressors may be separated according to their charge differences on either an alkaline continuous polyacrylamide gel (Figs. 1A and 2A) or a neutral discontinuous nondenaturing polyacrylamide gel (Fig. 3). Mutant RH54 and RH84 repressors have two additional negative charges per homodimer relative to wild type dimer and thus migrate to the anode faster, whereas mutant repressor GR85 has gained two positive charges and migrates more slowly. Mutant aporepressor TM44 is isoelectric with wild type and therefore exhibits wild type mobility. We chose mutant repressor RH84 for our initial studies of heterodimer formation with the WT aporepressor. Many of the conclusions reached with this mixture are applicable to mixtures with other mutant repressors. When purified samples of mutant and WT aporepressors were mixed and treated at 22°C, only the two homodimer bands are observed, as shown in Figure 1A, lane c. If the mixture was heated at 65°C for several minutes, a third heterodimer band appeared, which had an intermediate mobility reflecting its intermediate charge (see lanes d–h in Fig. 1A). Lanes a and b show that homodimers of WT or mutant protein do not produce this heterodimer band when treated individually at 65°C. Homodimer depletion was concomitant with heterodimer formation, as shown by the densitometry trace in Figure 1B and by the plot of the integrated values presented in Figure 1C. All combinations of repressor mixtures assayed yield a final equilibrium distribution of homodimer to heterodimer to homodimer of 1:2:1, which reflects the statistical distribution expected if association of individual polypeptide chains was random. The rate of heterodimer formation was similar for all combinations tested, implying that this property is independent of ability to negatively complement WT repressor when tested in vivo. Complete inhibition of heterodimer formation was achieved at high salt concentration, i.e., 0.6M NaCl. The high salt concentra-

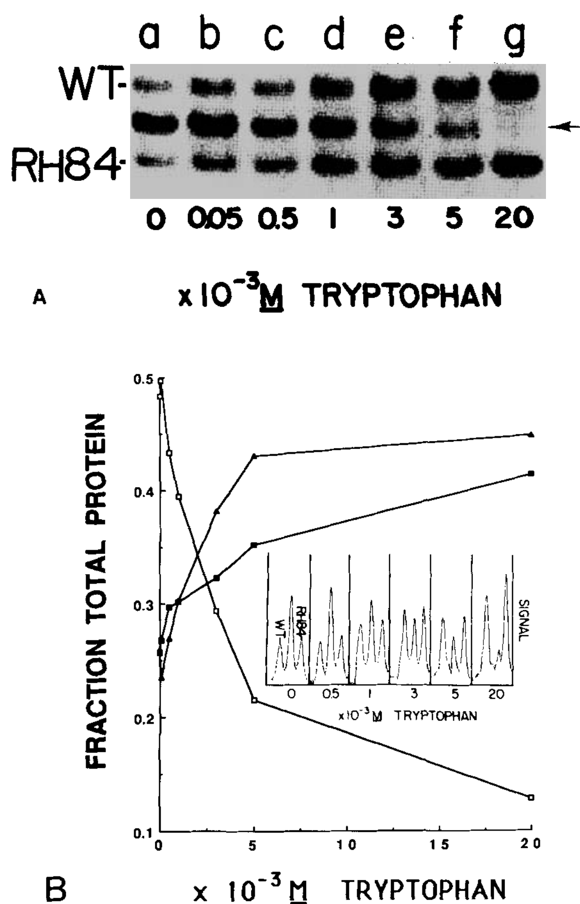


Fig. 2. **A:** Native alkaline continuous polyacrylamide gel stained with Coomassie brilliant blue R. Mixtures of WT and RH84 proteins were treated for 3 minutes at 65°C. The indicated concentration of L-tryptophan was added prior to heating. The arrow indicates the heterodimer band. **B:** Plot of protein content of each band as a function of the tryptophan concentration of the reaction mixture obtained from integrated values of densitometer traces: □, WT/RH84 heterodimer; ▲, WT homodimer; ■, RH84 homodimer. The value of 50% maximal inhibition of heterodimer formation ($K_{SI}$) was determined from this plot to be 2 mM tryptophan. The inset is the densitometer trace used to obtain this plot.

tion may prevent dimer dissociation by strengthening hydrophobic interactions between polypeptides of the dimer.

Figure 1A also shows the effect of varying temperature on heterodimer formation at a constant time of 3 minutes. Heterodimer was not detected when samples were incubated at 50°C for 3 minutes, although substantial formation occurs at 60°C and maximum levels were reached at 65°C. An incubation temperature of 80°C did not change the final distribution of dimer species but increased the rate, so that maximum formation occurs within 1 minute. Samples treated at 37°C for up to 8 hours failed to show significant heterodimer formation (data not shown).

## Effect of Tryptophan on Heterodimer Formation

Tryptophan was added to the mixture of RH84 and wild type aporepressors to examine its effect on heterodimer formation. The $K_S$ for tryptophan for wild type and RH84 repressors are 23 $\mu$M and 88 $\mu$M, respectively. The data in Figure 2A show that as the tryptophan concentration was increased, heterodimer formation was inhibited. A concentration of 2 mM L-tryptophan, determined from the plot presented in Figure 2B, was required to give one-half the maximal inhibition of heterodimer formation ($K_{SI}$). This concentration of tryptophan was required for all mixtures that included the WT aporepressor. Tryptophan was effective in inhibiting heterodimer formation whenever one homodimer species could bind corepressor. For example, RH54 by itself does not bind corepressor, but in a WT-RH54 mixture, the presence of 20 mM L-tryptophan completely inhibits the formation of the heterodimer.

Mutant RH54 and GR85 aporepressors do not bind tryptophan. When a mixture of these aporepressors was incubated at 65°C for 3 minutes in the presence or absence of 20 mM L-tryptophan, the rate of heterodimer formation was not affected (data not shown). Mutant aporepressors RH84 and TM44 bind L-tryptophan with dissociation constants of 88 $\mu$M and 39 $\mu$M at 25°C, respectively. When mutant TM44 and RH84 aporepressors were mixed, a somewhat higher concentration of tryptophan, 5 mM, than that required with a mixture of RH84 and WT aporepressors was required to give 50% inhibition of heterodimer formation (data not shown). These results correlate well with the corepressor binding properties of these mutant proteins and support the contention that inhibition of heterodimer formation results specifically from L-tryptophan binding.

## Ethanol-Induced Heterodimer Formation

The addition of ethanol to a homodimer mixture also results in heterodimer formation. The extent of heterodimer formation was dependent on the concentration of ethanol and on temperature. Complete heterodimer formation between mixtures of WT-RH84 or WT-RH54 was achieved with the addition of 26% ethanol at 22°C, while 20% ethanol was sufficient at 37°C (data not shown). As with thermal denaturation, the presence of 0.6 M NaCl prevented dimer dissociation induced by ethanol. These results reinforce the conclusion that heterodimer formation involves disruption of hydrophobic interactions between the polypeptides of *trp* aporepressor.

L-tryptophan prevented heterodimer formation induced by ethanol. When a WT-RH84 mixture was assayed at 37°C in 20% ethanol, 4 mM tryptophan completely prevented formation of heterodimer (data not shown). When 20 mM tryptophan was present in a 65°C sample assay, heterodimer formation was also prevented; however, the addition of 5% ethanol induced heterodimer formation.

## Dissociation of Heterodimer

We also examined dissociation of heterodimer to

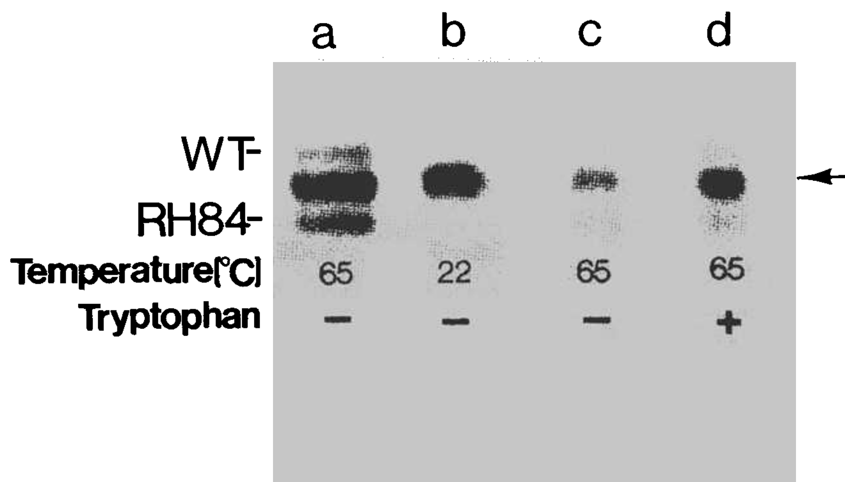


Fig. 3. Native neutral discontinuous polyacrylamide gel stained with Coomassie brilliant blue R. A heterodimer band was excised from an unstained native alkaline continuous gel, soaked in running buffer of the neutral gel for 15 minutes, and incubated at 65°C for 3 minutes (lanes a, c, and d). The sample in lane d was soaked in running buffer to which 20 mM tryptophan was added. The arrow indicates the heterodimer band.

form the respective homodimers. Bands representing heterodimer formed between WT and RH84 subunits were excised from samples run on an alkaline continuous gel and then incubated with and without L-tryptophan and electrophoresed on a neutral discontinuous gel. The results of this experiment are shown in Figure 3. In lane b, the heterodimer did not dissociate when samples were not heated. The emergence of homodimers from heterodimers occurred when samples were heated at 65 °C (lane c). If the gel slices were infused with 20 mM L-tryptophan, the thermal disruption of WT/RH84 heterodimer was retarded (lane d). We observed similar results with the WT/RH54 heterodimer. This latter species has only one wild type corepressor binding site, which apparently is all that is required for tryptophan inhibition of subunit dissociation.

### Effect of Tryptophan Analogs on Dimer Stability

The *trp* aporepressor shows significant affinity for a number of tryptophan analogs.[23] The $K_{SI}$ values reported in Figure 4 are the concentrations required for half-maximal inhibition of heterodimer formation with a mixture of WT and RH84 aporepressors, assayed following 3 minutes of incubation at 65 °C. The WT aporepressor binding constants for these analogs, obtained at 4 °C, were previously reported by Marmorstein, et al.[23] Aporepressor affinity for these ligands corresponds in general to the concentrations required to inhibit heterodimer formation. These results show a high specificity for L-tryptophan and certain analogs, since neither 20 mM D-tryptophan nor 20 mM L-phenylalanine prevent WT/RH84 heterodimer formation. The analog 5-methyltryptophan functions as a corepressor in vivo.[24] This analog binds to the WT aporepressor with greater affinity than L-tryptophan, $K_S$ of 3 $\mu$M at 4 °C.[23] As shown in Figure 4, the $K_{SI}$ for 5-methyltryptophan is one-tenth that of tryptophan. Indole-3-propionic acid (IPA) binds to *trp* aporepressor to yield an inactive complex.[25] IPA binds to the aporepressor 1.5 times more tightly than corepressor.[25] The $K_{SI}$ of IPA (0.8 mM) reflects this tighter binding relative to the $K_{SI}$ of 2 mM observed for L-tryptophan. At 6 mM, IPA completely inhibited heterodimer formation. The analogs tryptamine and

| Compound | Structure | $K_S$ ($\mu$M) | $K_{SI}$ (mM) |
|---|---|---|---|
| L-tryptophan | | 15 | 2 |
| 5-methyl-L-tryptophan | | 3 | 0.2 |
| indole-3-propionic acid | | 10 | 0.8 |
| indole | | 25 | 4 |
| tryptamine | | 41 | 4 |
| D-tryptophan | | 340 | NA |

Fig. 4. Half-maximal inhibition of WT/RH84 heterodimer formation ($K_{SI}$) by L-tryptophan and its analogs at 65°C. The $K_{SI}$ values obtained are listed with their structures alongside the dissociation constants ($K_S$) determined at 4°C by Marmorstein, et al.[23] The $K_{SI}$ values were derived from integration of densitometer traces much the same as shown in Figure 2B. The $K_{SI}$ value given for 5-methyl-L-tryptophan is based on the assumption that the D isomer does not bind. The range of L-tryptophan $K_{SI}$ values are within 15% of the value listed. NA stands for no affect at a concentration of 20 mM.

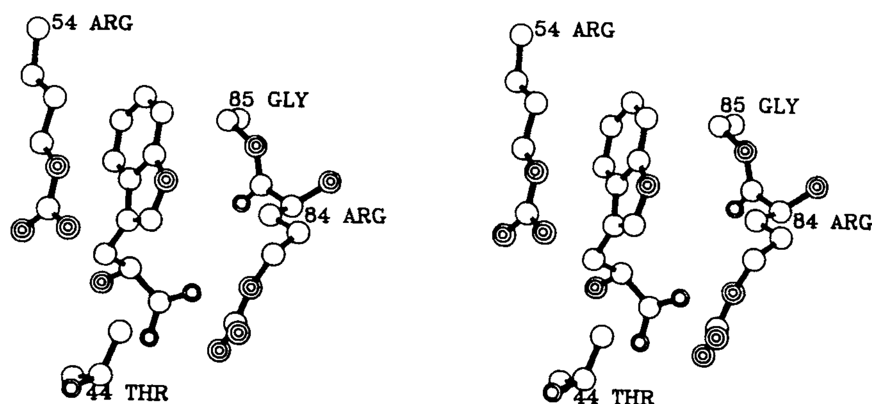


Fig. 5. Stereo view of one of the L-tryptophan binding sites in the *trp* repressor showing the position of the four amino acid residues surrounding the corepressor, tryptophan. The figure was generated by the drawing program of A. Lesk and K. Hardman.[29]

indole have $K_{SI}$'s of 4 mM in the heterodimer assay; these $K_{SI}$'s correspond to the aporepressor's weaker affinity for these species, 25 $\mu$M and 41 $\mu$M, respectively.[23]

## DISCUSSION

From the three-dimensional structure of the L-tryptophan-*trp* aporepressor complex (the *trp* repressor), the following amino acid residues are predicted to participate in specific binding of the corepressor, L-tryptophan: Thr 44, Arg 54, Arg 84, and Gly 85 (Fig. 5). Each of the two L-tryptophan binding sites in the *trp* aporepressor is formed from different segments of the two identical polypeptides. Thus, for a given corepressor binding pocket, Thr 44 is from one polypeptide chain, while Arg 54, Arg 84, and Gly 85 are from the second. Among the set of repressor mutants isolated previously, mutants were obtained that had amino acid changes at each of these positions.[12] The amino acid changes in these mutants are listed in Table I.

Repressor activities measured in vivo indicate that none of the mutant aporepressors is appreciably activated by tryptophan. Equilibrium dialysis analyses show that only two of the mutant repressors, TM44 and RH84, have near-normal tryptophan binding activity. Of the four mutant repressors studied, only these respond to tryptophan in vivo, and the response is slight. What is perhaps most striking about these results is that two repressors with only a modest reduction in tryptophan affinity nevertheless have very little repressor activity in vivo in the presence of tryptophan. Clearly these proteins are being saturated with corepressor, but are ineffective as repressors. Thus either Thr 44 and Arg 84 have other essential roles in repressor function, or the residues replacing them, Met 44 and His 84, respectively, disturb essential structural features of the protein while still allowing high-affinity tryptophan binding. Considering these changes separately, little can be said about the TM44 replacement. Since one surface of the tryptophan binding site is contributed by the DNA recognition helix, helix E, it is conceivable that the mutant Met residue displaces this helical region. Regarding the RH84 change, Arg 84 is within helix E, the helix that is presumed to interact specifically with the operator. Thus the RH84 change may cause localized positional changes in helix E that are detrimental to operator binding, in addition to slightly reducing tryptophan binding.

It is interesting to note that the GR85 mutation allows some repressor activity in vivo (Table I) despite the fact that we cannot detect tryptophan binding in vitro. Examination of a space-filling atomic model of the tryptophan binding site reveals that the base of the indole moiety of the corepressor is situated close to Gly 85. Thus one would predict that virtually any substitution at this position would inactivate the repressor. Perhaps the bulky arginine residue introduced at position 85 in GR85 causes conformational shifts in the protein segments normally responsible for corepressor binding. Other amino acid changes at position 85, GK85 (Gly→Lys) and GE85 (Gly→Glu) totally inactivate the repressor.[12] Further analyses of the effects of selected amino acid substitutions at these positions in the *trp* repressor may clarify the requirements for the critical conformational changes in the protein that normally accompany tryptophan binding.

The *trp* repressor forms a highly stable symmetric dimer composed of identical 107 residue polypeptides. Each polypeptide subunit is composed of six $\alpha$-helices (A–F), five of which make contacts with the apposing subunit.[11] The hydrophobic interactions between subunits of the *trp* repressor are extensive. If we assume an individual subunit has the same conformation found in the *trpR* dimer, then only 4% of its residues are excluded from solvent, atypical of the 20% found in monomers of most oligomeric proteins.[23] In the dimeric form, however, 18% of the residues of the repressor are buried.[23] This architecture presumably accounts for the marked dimer stability, since the

hydrophobic forces usually found in the interior of a globular protein are shared between subunits in the *trp* repressor. Nuclear magnetic resonance (NMR) analysis suggests that as temperature is increased from 25°C to 70°C the *trp* aporepressor retains its compact, folded structure and that its melting temperature is greater than 70°C.[26,27] In our study, however, wild type and mutant homodimers of *trp* repressor can exchange subunits to form heterodimers in vitro by heating at 65°C or by addition of 20% ethanol at 37°C, conditions that reduce intersubunit hydrophobic forces. The dissolution of hydrophobic bonds between polypeptide subunits of the *trp* repressor apparently permits the exchange of subunits between homodimers. This exchange leads to an equilibrium distribution of homodimers and heterodimer.

Even though the binding of tryptophan is thermally sensitive, significant binding activity is detectable at 65°C. An extrapolation of the van't Hoff plot presented by Arvidson et al.[22] suggests that aporepressor would bind tryptophan with a $K_S$ of 0.7 mM at 65°C. In this study, we find that upon binding L-tryptophan, or one of its analogs, the thermal stability of the repressor dimer increases significantly. Since the affinity for tryptophan is reduced at elevated temperatures, concentrations as high as 20 mM tryptophan are required to completely inhibit subunit dissociation. Significant retardation of subunit dissociation is observed when the heterodimer RH84/WT, which has one wild type site and one low-affinity site, is isolated and heated a second time in the presence of L-tryptophan. This result suggests that only one of the two corepressor binding sites per *trpR* dimer need be occupied by L-tryptophan in order to stabilize intersubunit binding.

The concentrations of tryptophan analogs required to inhibit heterodimer formation (Fig. 4) correlate well with their affinity for the aporepressor. However, the molecular details by which bound tryptophan or its analogs stabilize subunit interaction are unknown. First of all, the increased repressor stability in the presence of tryptophan would be expected to result primarily from interactions in the vicinity of the corepressor binding site, since crystallographic studies[9] have shown the absence of quaternary shifts along essentially the entire subunit interface when tryptophan binds. It is also reasonable to presume that the same mechanism of stabilization of the dimers applies to all of the analogs tested. The crystal structure of pseudorepressor supports this assumption, since the indole ring of IPA occupies approximately the same position as that of L-tryptophan in repressor, only it is "flipped over" 180° with respect to the long axis of the indole moiety.[28] Comparison of the structures of the analogs indicates that the indole moiety is sufficient to stabilize subunit interaction, i.e., the $\alpha$-carbon functional groups of bound tryptophan are not required for inhibition of subunit dissociation. The action of indole in preventing heterodimer formation presumably depends on its hydrophobic nature and/or its ability to form a hydrogen bond at its heterocyclic nitrogen. It has been proposed that the indole nitrogen of tryptophan forms a hydrogen bond with the aporepressor in a nonpolar environ of the corepressor binding pocket, resulting in large negative $\Delta S$ and $\Delta H$ values associated with its binding.[22] Structural analysis of repressor shows that the indole nitrogen of tryptophan forms a hydrogen bond with an ordered water molecule.[28]

Perhaps the hydrophobic nature of the indole moiety of tryptophan, IPA, tryptamine, or indole itself accounts for increased homodimer stability. When the corepressor binds, it displaces several water molecules and causes the microenvironment of the binding site to be less polar in the liganded species.[9] Since the corepressor binding site is formed between both subunits, the ability of indole to stabilize the dimer may be due to the addition of a hydrophobic patch to the subunit interface. It is also possible that corepressor binding may inhibit heterodimer formation indirectly by strengthening bonds that already exist between subunits. By shielding preexisting intersubunit electrostatic or ionic bonds from the solvent, tryptophan would significantly strengthen subunit interaction.

The fact that the analog concentration required for inhibition of heterodimer formation correlates with affinity for the aporepressor suggests that every feature that contributes to analog binding also influences stability. Thus the additional aporepressor-ligand interactions responsible for higher affinity binding of analogs such as 5-methyltryptophan and IPA would also be expected to increase stability of the analog-containing repressor.

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