Analysis of Heterodimer Formation by the *Escherichia coli trp* Repressor*

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Barry K. Hurlburt† and Charles Yanofsky‡

From the Department of Biological Sciences, Stanford University, Stanford, California 94305

The *trp* repressor of *Escherichia coli* is a dimeric DNA-binding protein that regulates transcription of several operons concerned with tryptophan metabolism. Although heterodimer formation between mutant and wild type subunits occurs readily in *vitro*, comparable heterodimers could be formed in *vitro* only under extreme conditions. To explain this difference we analyzed *trp* repressor dimer formation and dissociation using an *in vitro* transcription/translation system. Nascent wild type or mutant repressor polypeptides, synthesized in the presence of an excess of a second repressor, were invariably incorporated into heterodimers. In contrast, previously synthesized and assembled wild type dimers appeared to be refractory to dissociation, since they did not form heterodimers. However, previously synthesized mutant dimeric repressors that were defective in tryptophan binding readily dissociated and formed heterodimers. We noted that the ability of a dimeric repressor to dissociate under our conditions correlated inversely with its affinity for tryptophan. Consistent with this conclusion, we found that dissociation of the wild type aporepressor (no added tryptophan) was appreciably more rapid than dissociation of the tryptophan-saturated wild type repressor.

The *trp* repressor of *Escherichia coli* negatively regulates expression of four operons concerned with tryptophan transport and biosynthesis: *trp*EDCBA, arOH, mtr, and *trpR* (Squires et al., 1975; Gunsalus and Yanofsky, 1980; Zurawski et al., 1981; Heatwole and Somerville, 1991; Sarsero et al., 1991). Repression is presumed to result from competition between RNA polymerase and *trp* repressor for overlapping binding sites in the promoter/operator regions of the respective operons (Klig et al., 1988). The *trpR* gene encodes a 108-residue aporepressor polypeptide which, as a symmetrical dimer, binds two molecules of tryptophan noncooperatively to become the active, DNA-binding *trp* repressor (Joachimiak et al., 1983).

The three-dimensional structures of the aporepressor and the repressor have been determined by x-ray crystallographic (Schevit et al., 1985; Zhang et al., 1987) and NMR spectrographic studies (Arrowsmith et al., 1990, 1991). The structure of the crystalline *trp* repressor/operator complex also has been solved (Otwowski et al., 1988). These structural studies have shown that the *trp* repressor has an unusual dimeric interface. Four of the six a-helices of each polypeptide chain (helices A, B, C, and F) associate to form an intertwined dimerization domain, the "central core." Helices D and E, comprise helix-turn-helix DNA-binding motifs, or "reading heads," which are flexible extensions from the inflexible "core" domain, and presumably are poised for interactions with symmetrically disposed operator sequences. Two NH₂-terminal arms also extend from the core domain. The arms are disordered in all the structures analyzed, yet have been shown to contribute to repressor/operator interactions *in vitro* and repression in *vivo* (Carey, 1989; Arrowsmith et al., 1990; Hurlburt and Yanofsky, 1992). The structural features of the *trp* repressor and of the *trp* repressor/operator complex have recently been reviewed (Sigier, 1992).

In mutational studies with the *trp* repressor, two classes of dominant negative repressor mutants were isolated (Kelley and Yanofsky, 1985). The presence of a member of either class of mutant alleles in a cell producing the wild type repressor led to partial inactivation of the wild type repressor (Kelley and Yanofsky, 1985). Members of one class of dominant negative repressors had amino acid substitutions in the helix-turn-helix domain that presumably reduce operator binding while not affecting dimer formation. Members of the second class had substitutions of polypeptide residues that contribute to tryptophan binding and similarly do not reduce dimer formation. The reduced activity of co-expressed wild type and mutant repressors was thought to result from the formation of inactive or partially active heterodimeric repressors composed of one wild type polypeptide and one mutant polypeptide, and the concomitant reduction in the concentration of wild type homodimers.

Formation of heterodimers from mixed wild type and mutant repressor preparations has been studied *in vitro* (Graddis et al., 1988). When purified wild type and mutant repressors were mixed in a buffered salt solution and the mixture incubated at 37 °C for 1 h there was no detectable formation of a heterodimeric species. However, heterodimers were formed when repressor mixtures were subjected to treatments that disrupted the hydrophobic interactions that are presumed to stabilize the dimer. These treatments included incubation at an elevated temperature, 65 °C, or incubation in 20% ethanol at 37 °C. The presence of tryptophan during either treatment inhibited heterodimer formation. Tryptophan presumably stabilized existing repressor dimers that had functional tryptophan binding sites. In view of these observations and the interconnected polypeptide organization revealed by structural studies on the *trp* repressor, it was not obvious how heterodimer formation could occur so readily *in vivo*.
In an effort to explain heterodimer formation in vivo we performed heterodimer analyses using an in vitro transcription/translation system in which labeled trp repressor polypeptides were synthesized in the presence of an excess of an uncleaved trp repressor that had a different net charge. Our objective was to determine if newly synthesized repressor polypeptides would form heterodimers when they were synthesized in the presence of an excess of a second repressor. Under the conditions we used heterodimers were readily formed and detected. We determined that the presence of tryptophan and the tryptophan-binding capacities of the repressor species present play important roles in heterodimer formation and stability.

**EXPERIMENTAL PROCEDURES**

**Repressor Purification**—Wild type, TM44, RH54, and GR85 trp repressors were produced and purified as described elsewhere (Joachimiak et al., 1983; Paluh and Yanofsky, 1986). The *in vivo* and *in vitro* characteristics of the wild type and TM44, RH54, and GR85 mutant repressors have been described (Kelley and Yanofsky, 1985; Graddis et al., 1988). The purity of repressor preparations was assessed by SDS-polyacrylamide gel electrophoresis. Following Coomassie Brilliant Blue or silver staining, these repressor preparations were visually judged to be greater than 95% pure. Repressor concentration was determined spectrophotometrically using an extinction coefficient of 1.2 cm$^{-1}$ mg$^{-1}$ ml at 280 nm (Joachimiak et al., 1983).

**In Vitro Transcription/Translation of trp Repressor**—In *vitro* synthesis of trp repressor polypeptides was performed as previously described (Zubay et al., 1970). In *vitro* synthesis was programmed with high level expression plasmids (2 pg/reaction) containing wild type or mutant *trpR* genes (Paluh and Yanofsky, 1986). The *in vitro* transcription/translation reactions were incubated at 37 °C for 1 h. 25 μCi of [35S]methionine were included in each reaction to label the synthesized protein. To arrest translation, chloramphenicol was added to a final concentration of 2 mM.

Native Gel Electrophoresis, Autoradiography, and Western Analysis—Native slab gels, 16 × 14 cm, were run at 4 °C. Resolving gels were 10% acrylamide, 0.5% bisacrylamide, 237 mM Tris-CI, pH 8.5. Stacking gels were 3.125% acrylamide, 0.625% bisacrylamide, 40 mM Tris-phosphate, pH 6.9. Both gels were photo polymerized with riboflavin S'-phosphate and TEMED. The anode buffer was 63 mM Tris-CI, pH 7.5. Cathode buffer was 37.5 mM Tris-glycine, pH 8.9. Gels were run at 12 mA constant current for 4 h. Proteins were electrophoretically transferred to 0.1-μm nitrocellulose (Schleicher and Schuell, PH79) using the buffer system of Towbin et al. (1979) in a semidry transfer apparatus. Nitrocellulose filters were dried and exposed to Kodak XAR x-ray film overnight at room temperature. Nitrocellulose filters used in Western blot analysis were blocked with 5% bovine serum albumin in TBST (10 mM Tris-CI, pH 7.5, 0.9% NaCl, 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate)) and incubated with a 0.1% polyclonal, rabbit antiserum/TBST solution. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (GAR-HRP, Bio-Rad) was used to detect the primary antibody according to the manufacturer’s instructions using 4-chloro-1-naphthol as substrate.

Dimer Half-life Determination—Wild type repressor polypeptides were synthesized *in vitro* as described above. Chloramphenicol and phenylmethylsulfonyl fluoride (1 mM final concentration) was added. Nucleic acids were precipitated with streptomycin sulfate (1% final concentration). The supernatant was desalted by gel filtration chromatography into a buffer of 10 mM sodium phosphate, pH 7.6, 150 mM NaCl, 0.1 mM EDTA. GR85 repressor was added to 1 μM at t = 0. For the repressor dissociation determination, tryptophan was added to a final concentration of 300 μM. Homodimeric and heterodimeric species were identified following gel electrophoresis as described above.

**RESULTS**

**Wild Type and Mutant trpR Polypeptides Can Heterodimerize During In Vitro Synthesis**—The dominant negative effect of certain trpR mutant alleles on *in vivo* wild type repressor activity presumably reflects the formation of inactive or less active repressor heterodimers and a reduction of the concentration of wild type dimers (Kelley and Yanofsky, 1985). However, in previous studies in *vitro* heterodimer formation could be demonstrated only following drastic treatments, such as heating to 65 °C or incubation in the presence of 20% ethanol at 37 °C (Graddis et al., 1988). To examine heterodimer formation under physiological conditions, we used a coupled *in vitro* transcription/translation system to synthesize wild type trp repressor polypeptides or dominant negative mutant repressor polypeptides RH54 (Arg → His, position 54) or GR85 (Gly → Arg, position 85) from plasmid templates in the presence or absence of an excess of purified heterologous repressor. Residues Arg-54 and Gly-85 form part of the tryptophan-binding site of the trp repressor (Shevitz et al., 1985); the RH54 and GR85 mutant repressors have no detectable tryptophan binding (Graddis et al., 1988). Repressor synthesis was performed in the presence of [35S]methionine which radiolabeled newly synthesized repressor polypeptides. Homodimeric and heterodimeric proteins were resolved by native gel electrophoresis, and the protein species were transferred to nitrocellulose. An autoradiogram of a representative gel is shown in Fig. 1. When wild type or mutant repressor polypeptides were synthesized in the absence of a second repressor, a single radiolabeled species was observed (Fig. 1, lanes 1, 4, and 7). The mobility of each repressor species reflected its relative charge. Wild type repressor was arbitrarily assigned a relative charge of 0, and mutant RH54 and GR85 mutants had net charges of −2 and +2, respectively, relative to wild type repressor. The GR85 repressor did not resolve as well as repressors that were more negatively charged. Superrepressors EK13, EK18, and EK49 that have positive charges greater than +2 (Kelley and Yanofsky, 1985; Hurlbut and Yanofsky, 1990), could not be resolved using this gel system (data not shown).

When an excess (5–10 μM) of purified, heterologous repressor was added to the *in vitro* transcription/translation system, the electrophoretic mobility of the newly synthesized, radiolabeled repressor polypeptide was shifted to a position intermediate between that of the synthesized homodimer and that of the added homodimer (Fig. 1, lanes 2, 3, 5, 6, 8, and 9). The gel position of the added, unlabeled homodimeric repressor was determined by Western blot analysis following autoradiography and was always at the same position as the corre-

![Fig. 1. Analysis of heterodimer formation during in vitro synthesis of trp repressor polypeptides. Autoradiogram of the labeled repressor species that are formed following *in vitro* synthesis of wild type, RH54, and GR85 trp repressor polypeptides in the presence of the exogenous repressors indicated. Synthesized polypeptide refers to the polypeptide product of the trpR gene of the plasmid used as template in the *in vitro* transcription/translation reaction. Added protein indicates the source of the unlabeled, purified repressor (5–10 μM) that was present during synthesis. The concentration of synthesized repressor polypeptide was approximately 0.1 μM.](image-url)
Heterodimer Formation following in Vitro trp Repressor Synthesis—We next determined whether heterodimer formation was dependent upon the presence of heterologous repressor during in vitro polypeptide synthesis. Radiolabeled repressor polypeptides were synthesized using the in vitro transcription/translation system. Following synthesis, chloramphenicol was added to inhibit further translation, and an excess (5–10 μM) of heterologous repressor was added. The mixture was incubated at 37 °C for 1 h. In lanes 1–3 of Fig. 2, it can be seen that wild type trp repressor synthesized in vitro did not form heterodimers with added RH54 or GR85 repressors. However, when an excess of either wild type or mutant repressor was added to synthesized mutant trp repressor, nearly complete heterodimer formation was observed (Fig. 2, lanes 4–9). The latter results are indistinguishable from those shown in Fig. 1 (lanes 5, 6, 8, and 9) in which heterologous repressor was present during synthesis. The most likely explanation for this difference is that wild type repressor polypeptide synthesized in the presence of tryptophan forms a stable dimer, while the RH54 and GR85 repressor dimers formed in vitro have impaired tryptophan binding ability, therefore they are not stabilized by tryptophan to the same extent. Graddis et al. (1988) have in fact shown that tryptophan inhibits the ability of wild type repressor to form heterodimers.

Titrination of Wild Type and Mutant trp Repressors in Heterodimer Formation—The results of the experiments described above suggest that dominant negative trp repressors dissociate to monomers more readily than does the wild type trp repressor. To examine this possibility, we titrated the concentration of wild type, RH54, or TM44 (Thr → Met, position 44) mutant trp repressor required for heterodimer formation in the in vitro synthesis system. TM44 repressor was included in this experiment because it has a binding constant for tryptophan intermediate between that of the wild type and RH54 repressors (Table I). In Fig. 3A, RH54 repressor polypeptide was synthesized in the presence of increasing concentrations of purified wild type repressor. 50% heterodimer formation was observed at a wild type repressor concentration of approximately 150–160 nM. In Fig. 3B, RH54 repressor polypeptide was synthesized in vitro in the presence of increasing concentrations of TM44 repressor. 50% heterodimer formation occurred with approximately 80–90 nM added TM44. In Fig. 3C, wild type repressor polypeptide was synthesized in vitro in the presence of increasing concentrations of RH54 repressor. Approximately 40–50 nM RH54 repressor was required for 50% heterodimer formation. These results suggest that the dominant negative repressors RH54 and TM44 form dimers that are less stable than the wild type trp repressor and therefore they form heterodimers when present at lower concentrations than the wild type repressor. There is a negative correlation between the concentration of added repressor required for 50% heterodimer formation and the tryptophan binding affinity of the added repressor. These values are summarized in Table I.

**DISCUSSION**

Analysis of dominant negative mutants with altered trp repressors led to the conclusion that mutant and wild type trp repressor dimers exchange polypeptide chains in vivo to form inactive or less active heterodimeric species (Kelley and Yanofsky, 1985). To test this conclusion, heterodimer formation was studied in detail in vitro (Graddis et al., 1988). It was found that heterodimer formation did occur in vitro, but only when repressor mixtures were heat-treated or incubated in the presence of ethanol. These findings suggested that the trp repressor dimer, once formed, was very stable. Therefore it was not obvious how heterodimer formation could occur so readily in vivo. To examine the features of heterodimer formation more thoroughly we performed a series of experiments in which heterodimer formation was assayed in vitro.

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**Fig. 2. Analysis of the effects of addition of unlabeled repressor following synthesis of labeled trp repressor.** Labeled protein species were resolved by native gel electrophoresis. *Synthesized polypeptide* refers to the product of the trpE gene in the plasmid used in the in vitro transcription/translation reaction. *Added protein* indicates the particular unlabeled purified repressor (5–10 μM) added to the reaction mixture following addition of chloramphenicol to arrest translation. The concentration of synthesized repressor polypeptide was approximately 0.1 μM.

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**relative charge**

-2 +2 0
Heterodimer formation was determined in an in vitro transcription/translation system in which a trp repressor polypeptide was synthesized in the presence or absence of purified repressors with slightly different electrophoretic mobilities (Fig. 1). In vitro synthesized wild type repressor polypeptides were observed in heterodimeric complexes when synthesis proceeded in the presence of an electrophoretically distinguishable repressor. However, when a heterologous, mutant repressor was added following synthesis of wild type repressor, virtually no heterodimers were found (Fig. 2). This observation suggests that once a wild type repressor dimer has formed, there is very little dissociation to monomers, and therefore subsequent heterodimerization could not occur. This finding is in agreement with the conclusions of the previous in vitro heterodimerization study (Graddis et al., 1988). In contrast to our findings with wild type repressor synthesized in vitro, we observed that when wild type repressor was added following in vitro synthesis of a mutant repressor, nearly all of the radiolabeled, synthetic mutant repressor polypeptides were present as heterodimers (Fig. 2). Apparently mutant repressors readily dissociate to monomers while wild type repressors do not. This difference in dissociability was traced to tryptophan stabilization of dimers and heterodimers that have at least one wild type tryptophan binding site. Thus our results and those of Graddis et al. (1988) are in agreement that tryptophan binding reduces dissociation of a repressor dimer.

We examined heterodimer formation as a function of the concentration of exogenous repressor using wild type and mutant trp repressors as traps during in vitro synthesis of radiolabeled mutant or wild type repressor polypeptides (Fig. 3). The concentration of exogenous repressor required to achieve 50% heterodimer formation was inversely correlated with the affinity of the various exogenous repressors for tryptophan. Approximately four times more wild type repressor than RH54 repressor had to be added to achieve 50% heterodimer formation, using the same pair of repressors. RH54 repressor does not bind tryptophan measurably (Graddis et al., 1988). A lower concentration of TM44 repressor than wild type repressor was required for 50% heterodimer formation with synthesized RH54 repressor, in agreement with the fact that TM44 repressor does bind tryptophan, but less avidly than wild type repressor. In vivo RH54 repressor was one of the strongest negative complementing repressors; TM44 repressor was less effective (Kelley and Yanofsky, 1988).

![Fig. 3. Concentration dependence of heterodimer formation on exogenous trp repressor during in vitro repressor polypeptide synthesis. Autoradiogram of labeled repressors formed in vitro from synthesized wild type and mutant trp repressor polypeptides; protein species were resolved by native gel electrophoresis. A, RH54 repressor polypeptides synthesized in the presence of increasing concentrations of wild type repressor. B, RH54 repressor polypeptides synthesized in the presence of increasing concentrations of TM44 repressor. C, wild type repressor polypeptides synthesized in the presence of increasing concentrations of RH54 repressor. The asterisks mark the concentrations of exogenous repressor that yield 50% heterodimers.](image-url)
residues that would be exposed to solvent upon dimer dissociation. Although our results on repressor dissociation cannot be compared directly to the NMR data, they correlate qualitatively.

The trp repressor of E. coli has an atypical dimeric structure (Sigler, 1992). Its identical polypeptide chains intertwine to form an extensive dimer interface. Thus helices A, B, C, and F of the two chains associate to form a stable, globular core domain from which two helix-turn-helix domains extend. The helices that form the helix-turn-helix motifs are flexible in solution (Arrowsmith et al., 1990, 1991) and occupy different positions in two different types of repressor crystals (Schevitz et al., 1985; Zhang et al., 1987; Lawson et al., 1988). Tryptophan binding to the repressor reduces the flexibility of the latter helices (Zhang et al., 1987; Lawson et al., 1988; Arrowsmith et al., 1991). In the present and previous heterodimerization studies (Graddis et al., 1988) bound tryptophan was observed to stabilize the dimeric structure. Since each tryptophan binding site in the trp repressor is composed of residues from two polypeptide chains, helices C and E from one chain and helix B from the other, bound tryptophan probably stabilizes the dimer by its interactions with residues of both chains.

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REFERENCES