Molecular Basis of Transcriptional Antiactivation

TraM DISRUPTS THE TraR-DNA COMPLEX THROUGH STEPWISE INTERACTIONS

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Conjugative transfer of Agrobacterium Ti plasmids is regulated by TraR, a quorum-sensing activator. Quorum dependence requires TraM, which binds to and inactivates TraR. In this study, we showed that TraR and TraM form a 151-kDa stable complex composed of two TraR and two TraM dimers both in vitro and in vivo. When interacted with TraR bound to tra box DNA, wild-type TraM formed a nucleoprotein complex of 77 kDa composed of one dimer of each protein and DNA. The complex converted to the 151-kDa species with concomitant release of DNA with a half-life of 1.6 h. TraR in the complex still retained tightly bound autoinducer. From these results, we conclude that TraM interacts in a two-step process with DNA-TraR to form a large, stable antiactivation complex. Mutagenesis identified residues of TraR important for interacting with TraM. These residues form two patches, possibly defining the binding interfaces. Consistent with this interpretation, comparison of the trypsin-digested polypeptides of TraR and of TraM with that of the TraR-TraM complex revealed that a trypptic site at position 177 of TraR around these patches is accessible on free TraR but is blocked by TraM in the complex. From these genetic and structural considerations, we constructed three-dimensional models of the complex that shed light on the mechanism of TraM-mediated inhibition of TraR and on TraM-mediated destabilization of the TraR-DNA complex.

Agrobacterium tumefaciens, the causative agent of plant tumors called crown galls, utilizes a LuxR/LuxI-type quorum-sensing system to regulate the conjugative transfer of its tumor-inducing (Ti) plasmids (1, 2). This system is composed of the transcriptional activator TraR and the acyl-homoserine lactone synthase TraI. TraR binds to its cognate autoinducer, N-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL), produced by TraI and activates the expression of three operons that are responsible for Ti plasmid conjugation (3–6). Unique to this system is a negative regulator, TraM, that inhibits activation of target genes by TraR (7, 8). TraM is associated with all TraR-dependent conjugative transfer systems, including those of plasmids of Sinorhizobium meliloti (9), Rhizobium leguminosarum (10), and Rhizobium sp. NGR234 (11). Previous studies with nopaline- and octopine-type Ti plasmids as well as plasmids of Sinorhizobium and Rhizobium species all speak to the fact that null mutations of TraM yield donors that are hyperconjugative (7–11). Such mutants still require the autoinducer, but expression of the tra regulon occurs in a population-independent fashion (2, 7, 8). Thus, TraM is not required for autoinduction but is essential for the quorum-dependent nature of the regulation of plasmid transfer (2).

TraM exerts its regulatory activity by binding very tightly to TraR (12, 13), and interaction with TraM prevents TraR from binding its promoter recognition element (12, 13). Deletion analysis located the TraM binding region to the C-terminal 92 residues of TraR (residues 172–234) (12). Mutational analysis of TraR identified five residues, Pro-176, Arg-215, Leu-182, Ala-195, and Arg-213 or Met-213 or Arg-215, that are required for TraM binding (12, 14). Consistent with the deletion analysis, these residues are located near the helix-turn-helix motif at the C terminus of the activator. However, as mapped on the crystal structure of TraR, these residues are scattered and do not form an obvious surface patch (15, 16).

Based on genetic, biochemical, and x-ray crystallographic studies, TraM exists as a highly compacted homodimer (15–17). In the crystal structure, the two monomers are arranged in a head-to-tail fashion, in which each protomer presents a T-shape structure consisting mainly of the two anti-parallel α helices (15, 16). Deletion analysis indicates that these two α helices are involved in dimerization and that, except for the first 10 N-terminal residues, the rest of TraM is essential for its antiactivator activity (17). Extensive substitution mutagenesis of TraM identified eight residues, Leu-29, His-40, Arg-41, Leu-54, Tyr-72, Val-86, Gly-94, and Pro-97, that are important for binding TraR in vitro as well as for inhibiting TraR activity in vivo (13, 14, 17). All of these residues on the two protomers are surface-exposed and face exclusively on one side of the dimer. None are involved in the dimeric interface (16).

Although studies based on Far Western and surface plasmon resonance analyses suggest that TraR and TraM interact with high affinity, there is little information concerning the nature of the complexes they form (12, 13). Chen et al. have suggested that the TraM dimer dissociates during its interaction with TraR and that the resulting complex contains one monomer of TraM and one monomer of TraR (15). However, a second group proposed a model based on size exclusion chromatography and dynamic light scattering, in which the complex is formed from two dimers of TraR and two dimers of TraM (16). In a recent report from Chen et al. (18), they found that TraM2, an ortholog of TraM encoded in the genome of A. tumefaciens strain A6, forms a hetero-octomer with TraR in a stoichiometry of 1:1.
Characteristics of TraM-TraR Interactions

of 1:1. In the present study, we show that in the absence of DNA, TraR, and TraM form a stable ~151-kDa complex containing two dimers of TraR and two dimers of TraM. We also show that TraM interacts with DNA-bound TraR to form an intermediate nucleoprotein complex containing one dimer of TraR, one dimer of TraM, and a molecule of dsDNA. This nucleoprotein complex rearranges to form the stable ~151-kDa complex with the release of the DNA. We also provide information regarding the contact interfaces of TraR and TraM. Finally, using this information, we propose three-dimensional models of the two complexes formed by TraR and TraM.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media—Bacteria used in this study included Escherichia coli BL21(DE3) (T7 promoter expression host) (Novagen) and Agrobacterium tumefaciens strain NTL4, a Ti plasmid-cured derivative of C58 (19). Strain NTS2 is a lon mutant of strain NTL4 (20).

Strains of E. coli were grown in Luria-Bertani broth (LB) or a minimal medium with glucose as sole carbon source (21). Strains of A. tumefaciens were grown in MG/L, nutrient broth (Difco), or ABM minimal medium (22, 23). Antibiotics were used at the following concentrations: ampicillin (100 or 200 μg/ml), chloramphenicol (34 μg/ml), kanamycin (50 μg/ml), carbenicillin (50 μg/ml), and tetracycline (2 μg/ml for A. tumefaciens and 10 μg/ml for E. coli). When necessary, iso-propyl-β-D-thiogalactopyranoside or synthetic 3-oxo-C8-HSL (Sigma) was added to cultures at concentrations as described throughout.

Overexpression, Purification, and Cleavage of Proteins—Active TraR containing 3-oxo-C8-HSL was overexpressed from pETR (pET17b::traR) in E. coli strain BL21(DE3) cultured in the presence of this signal at 28 °C as described previously (24). The protein was purified as described previously with the following modifications (24). Briefly, TraR first was eluted from HiPrep™ 16/10, a prepacked Heparin FF column, using a 250-ml gradient of NaCl from 0.3 to 1 M. Fractions containing TraR were pooled and diluted with three volumes of TEDG buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 100 mM 3-oxo-C8-HSL). The salt-diluted sample was purified to over 98% homogeneity by two sequential passages through a Mono S column. The purified TraR protein was dialyzed against and aliquoted in TNEDG buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 100 mM 3-oxo-C8-HSL) and stored at −80 °C.

His6-tagged TraM and its mutant derivatives were overexpressed from pET14b::traM (14) in E. coli strain BL21(DE3) grown at 37 °C in LB. The His6-tagged proteins were purified using a nickel affinity column as described previously (12). Following purification, the proteins were treated with bionitinated thrombin for 2 h to remove the epitope tag using a thrombin cleavage capture kit (Novagen). The resulting thrombin-cleaved TraM protein contains three extra residues, Gly-Ser-His, at the N terminus. Following further purification using nickel affinity column chromatography, the processed TraM was dialyzed against TNEDG buffer containing 30% glycerol and stored at −20 °C.

Gel Filtration Chromatography of Purified Proteins—Samples containing TraR, TraM, the 18-bp tra box DNA (ACGT-GCAGATCTGACGCT), or their mixtures were chromatographed on Superdex 200HR using an AKTA fast protein liquid chromatography system as described previously (15). The column was developed with 36 ml of TNEDG buffer containing 300 mM NaCl at a flow rate of 0.35 ml/min. The elution profiles were calibrated with a set of molecular weight standards of proteins purchased from Sigma.

Gel Filtration Chromatography of Cell Extracts—Five hundred-ml cultures of A. tumefaciens NTS2 (20) expressing only TraR from pKMA1 (7) or both TraR from pKMA1 and TraM from pKKTM (17) were harvested by centrifugation and resuspended in 10 ml of extraction buffer TNEDG containing 0.5 mg/ml lysozyme, DNase, and RNase. The cells were broken by a combination of sonication and three passages through a French pressure cell. The total cell extracts were cleared by high speed centrifugation for 2 h. Ten replicates of 1-ml samples of the resultant supernatant were subjected to our standard gel filtration chromatography as described above. The elution fractions were collected in a volume of 1 ml. Corresponding individual fractions from the 10 chromatographic separations were combined and treated with 10% trichloroacetic acid to precipitate the proteins. Following a brief wash with cold ethanol, the trichloroacetic acid precipitates were dried by SpeedVac and dissolved in 200 μl of 1× SDS-PAGE loading buffer for Western analysis using anti-TraR and anti-TraM antibodies.

Gel Mobility Shift Assay—A 251-bp digoxigenin-labeled DNA fragment containing the entire intergenic region between the traAFB and traCDG operons of pTiC58 was produced by PCR amplification followed by digoxigenin labeling (Roche Applied Science). Gel mobility shift assays were performed as described previously (12). Samples of TraM or its mutant, TraM Q82A, at concentrations of 25 nM, 250 nM, 2.5 μM, 5 μM, and 7.5 μM were incubated with TraR for 30 min before or after the binding of TraR to DNA. In the DNA binding reaction mixtures, the concentrations of labeled DNA and active TraR were 1.6 and 25 nM, respectively.

Western and Far Western Analysis—The stability and expression levels of TraR mutant proteins in vivo and the amount of purified TraR protein spotted on membrane strips were determined by Western analysis using anti-TraR antiserum as described previously (12). Interactions between purified TraM and TraR proteins on membrane strips or separated by SDS-PAGE were assessed by Far Western analysis using anti-TraM antiserum as described previously (12).

Amino Acid Analysis—Amino acid analysis was performed using a Hewlett Packard AminoQUANT II system by the Protein Chemistry Laboratory at Texas A & M University. Three 20-μl volumes of TraR-TraM complex containing about 5 μg of total protein were used for each analysis.

Extraction of Acyl-HSL from Whole Cells—Cells of A. tumefaciens were washed thoroughly by centrifugation and were broken by a combination of sonication and three passages through a French pressure cell. The broken cell preparation was extracted with ethyl acetate three times as described previously (25), and the resulting extracts containing 3-oxo-C8-HSL were combined and concentrated to dryness using a Vacufuge.
The residues were redissolved in 20 μl of high pressure liquid chromatography grade ethyl acetate, and 5-μl samples were subjected to TLC analysis.

**Extraction of Acyl-HSL from Purified TraR Protein**—One hundred-μl samples of TraR, TraR-TraM complex, and a mixture of TraR with TraM were dialyzed in 100 ml of TNEDG buffer in the absence of 3-oxo-C8-HSL for 3 days with six changes of buffer. Following dialysis, the protein samples were extracted three times with 100-μl volumes of ethyl acetate, and the organic phases were pooled and taken to dryness. The resulting residues were redissolved in 20 μl of ethyl acetate, and 5-μl volumes of ethyl acetate, and methanol with 0.1% formic acid. Following dialysis, the polypeptides in the digests were analyzed on a Micromass Q-TOF Ultima, a hybrid quadrupole time-of-flight mass spectrometer by the Mass Spectrometry Laboratory in the school of Chemical Science at the University of Illinois at Urbana-Champaign.

**RESULTS**

**TraR Forms a Stable 151-kDa High Molecular Weight Complex with TraR**—To identify the complexes formed between TraR and TraR in solution, we subjected purified active TraR, purified TraM, and mixtures of the two to gel filtration chromatography. TraR, in dimer form, elutes at a position corresponding to a molecular size of 52 kDa (24), whereas TraR, also in dimer form, elutes with an apparent molecular mass of 25 kDa (Table 1) (17). The TraM-TraR complexes, prepared by co-incubation of purified TraM and TraR proteins at a 1:1 molar ratio for 30 min, co-eluted as a broad, biphasic set of high molecular weight peaks (Fig. 1A, I and II), indicating that more than one species of complexes are formed under these conditions (13). However, as the molar ratio of TraM to TraR in the mixture was increased, Peak I decreased, and Peak II, eluting between 11.5 and 12.5 ml, corresponding to a size of 151 kDa, increased proportionally (Fig. 1, B and C). Increasing the co-incubation time from 30 min to overnight did not significantly improve the proportion of the Peak II complex in the mixtures of TraM and TraR under any given molar ratio of reactants (data not shown), suggesting that 30 min of preincubation is sufficient to reach equilibrium in the interaction between the two proteins. In addition, co-incubation of the two proteins at all of the three molar ratios tested (1:1, 2:1, and 5:1) often yielded very high molecular weight aggregates (Peak 0) that eluted at the void volume of the column (Fig. 1, A–C).

We combined and concentrated the fractions eluting at 10–11 ml (Peak I) and 11.5–12.5 ml (Peak II) and reexamined the behavior of these complexes on a gel filtration column following an overnight incubation at 4 °C. The resulting elution profiles indicated that the species in Peak II, corresponding to a molecular size of 151 kDa, is a stable complex (Fig. 1E and Table 1), whereas the higher molecular weight complex in Peak I partially converts to the 151-kDa species (Fig. 1D).

**TABLE 1**

| Reaction mixture | Peak positiona | Predicted sizeb | Substrates in peaks | Calculated sizec |
|------------------|----------------|-----------------|---------------------|-----------------|
| TraR  | TraM  | DNA  | ml  | kDa  | TraR<sup>f</sup>  | 53   |
| +  | −  | −  | 14.37  | 45   | TraR<sub>2</sub>  | 23   |
| +  | +  | −  | 14.9  | 25   | 18-bp dsDNA  | 11   |
| −  | +  | +  | 15.5  | 20   | 2TraR<sub>2</sub>-2TraM<sub>2</sub>  | 153  |
| +  | +  | −  | 13.7/15.55  | 55/20 | TraR<sub>2</sub>-dsDNA/dsDNA  | 64/11 |
| +  | +  | +  | 11.94/13.14/15.54  | 151/77/20 | 2TraR<sub>2</sub>-2TraM<sub>2</sub>/TraM<sub>2</sub>−TraR<sub>2</sub>-dsDNA/dsDNA  | 153/87/11 |
| +  | Q82A  | +  | 15  | 24   | 2 TraR<sub>2</sub>-2(TraMQ82A)  | 23   |
| +  | Q82A  | −  | 11.94/13.35  | 151/69 | TraR<sub>2</sub>-(TraMQ82A)  | 153/76 |
| +  | Q82A  | +  | 13.15/15.55  | 77/20 | TraR<sub>2</sub>-(TraMQ82A)−dsDNA/dsDNA  | 87/11 |

* The average from three independent experiments with a variation of ±0.05.
* Predicted based on the elution position of the gel filtration molecular markers.
* Calculated based on the amino acid sequences of proteins.
* Represents the active dimeric TraR complexed with AAl.
* The elution position of the stable TraM-TraR complex isolated from the multiple complexes formed between TraM and TraR.
To investigate the biological relevance of these antiactivation complexes in vivo, we assessed the characteristics of the TraR-TraM complexes in A. tumefaciens strain NTS2 (20) harboring pKMA1, a derivative of pTiC58 that lacks traM but expresses TraR and produces 3-oxo-C8-HSL constitutively (7), and pKKTM, a pKK38 derivative expressing TraM from the trc promoter (17). TraR was detectable in its monomer (Fig. 2A, lanes 6 and 7), and dimer (lane 3) forms in lysates from NTS2(pKMA1, pKK38), which lacks traM. No higher molecular weight forms of TraR were observed in lysates from this strain (Fig. 2A, lanes 1 and 2). However, strain NTS2(pKMA1, pKKTM), which co-expresses both TraR and TraM, yielded a TraR-TraM complex (Fig. 2, B and C, lanes 1 and 2) that elutes at the same position as the ~151-kDa complex, as assessed by gel filtration chromatography followed by SDS-PAGE. This result suggests that the 151-kDa TraR-TraM complex can form in vivo under the conditions tested.

**Stoichiometry of TraM and TraR within the Complexes**—Six possible stoichiometries of TraM to TraR could account for the 151-kDa complex: 145,455 kDa, four dimers of TraM plus one dimer of TraR (4:1); 122,455 kDa, three dimers of TraM plus one dimer of TraR (3:1); 149,182 kDa, three dimers of TraM plus two dimers of TraR (2:1); 152,908 kDa, two dimers of TraM and two dimers of TraR (1:1); 129,909 kDa, one dimer of TraM plus two dimers of TraR (1:2); and 183,363 kDa, one dimer of TraM plus three dimers of TraR (1:3).

Compositional analysis, commonly expressed in mole percentage (mol %) of each amino acid relative to all other residues, provides a unique distribution profile of all amino acids in a particular protein. As such, quantifying amino acid composition can be used to characterize protein species. We determined the stoichiometry of TraM to TraR in the ~151-kDa species by quantifying the amino acid composition of the complex using amino acid analysis. Of the 19 amino acids analyzed, 10 of them are stoichiometry-sensitive with respect to the six possible TraM/TraR ratios. Therefore, we selected these 10 amino acids and used their compositional profiles from amino acid analysis to determine the stoichiometry of TraM/TraR in the complex. As shown in Table 2, the mol % of the 10 amino acids was in best agreement with a 1:1 TraM/TraR stoichiomet-
The 151-kDa TraM-TraR complex is composed of two dimers of TraM and two dimers of TraR.

TraM Forms an Unstable Complex with DNA-bound TraR—Based on previous mutational analysis, TraM binds to TraR at a region close to the helix-turn-helix DNA-binding domain located at the C terminus of the activator (12). This observation suggests that TraM might interact with DNA-bound TraR to form an intermediate nucleoprotein complex. Consistent with this hypothesis, we previously reported formation of a ternary complex of TraM-TraR-DNA, which migrates slower than the TraR-DNA complex in gel mobility shift assays (12, 17). The appearance of this complex relies on the concentration and the order in which TraM is added to the mixtures (Fig. 3) (12, 17). When TraR was incubated with DNA followed by addition of TraM (Fig. 3A, lanes 3–7), the ternary complex of TraM-TraR-DNA began to appear at a TraM/TraR molar ratio of 100:1 and remained constant up to a molar ratio of 500:1. However, when TraR was first reacted with TraM and then incubated with DNA, only a trace amount of this nucleoprotein complex was detected at molar ratios of TraM to TraR of 10:1 and 100:1, and none of this complex was detected at higher molar ratios (Fig. 3B).

To characterize this nucleoprotein complex, we incubated TraM with DNA-bound TraR in solution and analyzed the complexes and their components formed in the resulting mixture on a gel filtration column (Fig. 4 and Table 1). The 18-bp tra box DNA fragment eluted at 15.5 ml (Fig. 4A and Table 1), whereas the DNA-TraR complex eluted at a volume corresponding to a molecular size of 55 kDa (Fig. 4B and Table 1). As expected, incubation of TraM with the mixtures of TraR and DNA yielded a complex that eluted at a position corresponding to a size of 151 kDa (Fig. 4, C and D, and Table 1). However, under these conditions, a new complex was observed that eluted at a position corresponding to ~77 kDa.

TABLE 2
Composition of amino acids in the stable TraM-TraR complex (151 kDa) as measured by amino acid analysis (AAAn) compared with those calculated by sequences of TraM and TraR under different ratios of TraM to TraR

| Amino acid | Calculated mol % at TraR/TraM ratios of mol % by AAAa |
|------------|-----------------------------------------------------|
|            | 1:4 | 1:3 | 1:2 | 1:1 | 2:1 | 3:1 |
| Asn + Asp  | 6.93 | 7.17 | 7.51 | 8.08 | 8.52 | 8.71 | 8.02 ± 0.03 |
| Glu + Gln  | 11.3 | 11.03 | 10.7 | 10.2 | 9.76 | 9.60 | 9.67 ± 0.20 |
| Ser        | 6.78 | 6.62 | 6.38 | 5.99 | 5.68 | 5.56 | 6.08 ± 0.04 |
| Ala        | 11.1 | 11.4 | 11.9 | 12.6 | 13.14 | 13.38 | 12.1 ± 0.01 |
| Arg        | 5.86 | 6.07 | 6.38 | 6.89 | 7.28 | 7.45 | 6.39 ± 0.04 |
| Phe        | 2.16 | 2.39 | 2.73 | 3.29 | 3.73 | 3.91 | 3.21 ± 0.02 |
| Leu        | 10.9 | 10.66 | 10.3 | 9.58 | 9.06 | 8.84 | 9.54 ± 0.06 |
| Pro        | 3.85 | 3.68 | 3.42 | 2.99 | 2.66 | 2.53 | 3.00 ± 0.17 |

a The 10 stoichiometry-sensitive amino acids of the 19 amino acids analyzed.

The 77-kDa complex is composed of one dimer of TraM, one dimer of TraR, and also DNA (Fig. 4D). This large complex remained stable following incubation at 4 °C for as long as several days (data not shown).

To test the stability and fate of the ~77-kDa complex described above, we isolated the fractions containing this species and incubated them at 4 °C. Samples were taken at intervals and subjected to gel filtration analysis. As shown in Fig. 4, E–G, the complex is unstable and gradually converts to the 151-kDa complex and free DNA. Quantifying the increasing peak area of the ~151-kDa complex in Fig. 4, E–G, we estimated that the half-life of the ~77-kDa complex is ~1.6 h.

Fractions containing the ~77-kDa nucleoprotein complex of TraM-TraR-DNA, were subjected to amino acid analysis. As expected, the compositional percentage of the 10 amino acids fit best with a stoichiometry of 1:1 (TraM/TraR), suggesting that the ~77-kDa complex is composed of one dimer of TraM, one dimer of TraR, and a copy of double strand tra box DNA (data not shown).

A Substitution Mutant of TraM Binds to TraR but Does Not Form the Stable Complex as Efficiently as the Wild-type Antiactivator—Q82A, a substitution mutant of TraM from an octopine-type Ti plasmid, binds TraR in vitro but does not inhibit activation by TraR in vivo (13). Similarly, the Q82A substitution mutant of TraM from the nopaline-type Ti plasmid bound TraR reasonably well (about 2–4-fold less affinity than the wild-type TraM), as assessed by a Far Western assay (data not shown). However, as assessed by gel mobility shift assays, the Q82A mutant showed a 500-fold reduced ability to disrupt TraR-DNA complexes (Fig. 3). When tested in vivo in our reporter system, the Q82A mutant only moderately inhibited TraR-mediated activation of the traG::lacZ reporter (Table 3).

We used gel filtration chromatography to investigate the oligomeric state of this mutant protein as well as the complexes formed upon its interaction with TraR or with DNA-bound TraR. Like the wild-type protein, the mutant TraM protein

FIGURE 3. TraM disrupts the TraR-DNA complex and inhibits free TraR from binding DNA. Purified active TraR (25 nM), was preincubated with a sample of the 251-bp digoxigenin-labeled tra box probe DNA (1.6 nM) for 30 min. TraR (lanes 3–7) or its mutant TraRQ82A (lanes 8–12) was then added, and the mixture was incubated for another 30 min (A). In B, TraR was first reacted with TraM or its mutant for 30 min followed by the addition of DNA and another 30-min incubation at concentrations described in the legend to A. The reaction mixtures were subjected to native gel electrophoresis, and the probe DNA was detected as described under “Experimental Procedures.” Lane 1, DNA; lane 2, TraR and DNA; lanes 3–12, DNA, TraR, and TraM or TraM Q82A at concentrations yielding molar ratios to TraR as indicated. Bands represent free DNA (I), TraR-DNA complex (II), and TraM-TraR-DNA complex (III).
eluted from the gel filtration column as a dimer in solution (Fig. 5A). However, in contrast to wild-type TraM, a mixture of TraR and the mutant TraM yielded two major complexes (Fig. 5A and Table 1). One, corresponding to a size of around 151 kDa, is equivalent to the stable complex formed between wild-type TraM and TraR proteins. The second eluted at a new position corresponding to a size of 69 kDa, which accounts for one dimer of TraR and one dimer of TraM Q82A (Table 1 and Fig. 5A). In addition, the Q82A mutant protein formed a ~77-kDa unstable nucleoprotein complex with DNA-bound TraR, but this complex was converted back to TraR-DNA with the concomitant release of free TraM in less than 30 min (Table 1 and Fig. 5, B and C). Moreover, although the Q82A mutant can interact with TraR dimers to form a 69-kDa complex or interact with DNA-bound TraR to form a 77-kDa complex, these intermediate complexes do not efficiently isomerize to form the 151-kDa complex (Fig. 5). These data imply that whereas the Q82A mutant binds to TraR, the lower affinity prevents the efficient conversion to the stable 151-kDa complex.

**Formation of the Antiactivation Complexes Does Not Release 3-oxo-C8-HSL from TraR**—TraR in its active dimer form contains two tightly bound molecules of its cognate autoinducer 3-oxo-C8-HSL (28–30). Loss of this signal results in conversion of the activator from a dimer to a monomer and consequent loss of activity (24, 31). We considered the possibility that interaction of TraM with TraR results in a conformational change in the activator protein with loss of the bound acyl-HSL ligand. To test this hypothesis, we dialyzed highly purified active TraR or the same amount of TraR mixed with purified TraM at a 1:1 molar ratio as described under “Experimental Procedures.” The resulting protein samples were assayed for bound acyl-HSL by extraction with ethyl acetate followed by thin layer chromatography. As shown in Fig. 6A, interaction with TraM had no detectable effect on the amount of 3-oxo-C8-HSL retained by TraR following dialysis. Consistent with this result, extraction of the purified stable 151-kDa TraM-TraR complex with ethyl acetate yielded 3-oxo-C8-HSL at a level similar to that of a sample of free TraR at the same concentration (Fig. 6B).

### TABLE 3

| TraM allele | traG::lacZ reporter | β-Galactosidase activity | Inhibition |
|-------------|---------------------|--------------------------|-----------|
| None        | 2338 ± 600          | NA*                     | 100%      |
| Wild type   | 4 ± 0.5             | 584                      | 500%      |
| Q82A        | 26 ± 6.0            | 90                       | 90%       |

* *TraM or its mutant was expressed from the P~14promoter in pZLQ.

* traG::lacZ fusion reporter with TraR supplied in pEKTR2-I to measure TraR activation activity in A. tumefaciens.

*Expressed as units/10⁹ colony-forming units (cfu) as described under “Experimental Procedures.”

* β-Galactosidase activity of the traG::lacZ reporter in the strain expressing TraR alone divided by that of strains expressing both TraR and TraM or the TraM mutant.

* Not applicable.
Characteristics of TraM-TraR Interactions

To probe the interaction interfaces on TraR and on TraM, we examined the trypsin sensitivity of the 151-kDa complex in comparison with those of the two individual proteins. Fifty-μl volumes of TraR, TraM, and the TraR-TraM complex at a concentration of about 1.5 mg/ml were digested with trypsin at a final concentration of 0.04 mg/ml for 1 min, 30 min, 1 h, 2 h, and overnight, respectively. SDS-PAGE analysis of these treated samples indicates that the digestion patterns are relatively stable between 30 min and 2 h of treatment (data not shown). Based on this information, we selected 1-h trypsin-digested samples for analysis by mass spectrometry to identify the polypeptide species occupying the protein bands observed on SDS-polyacrylamide gels.

Free TraR yielded as the most abundant species a fragment at 9217.0 Da corresponding to residues 92–177 of TraR. However, this fragment was barely detectable in the digests of the 151-kDa complex (Fig. 7, A and C). A second fragment at 15820.0 Da corresponding to residues 92–234 of TraR was dominant in the digests of the 151-kDa complex but was barely detectable in the digests of free TraR (Fig. 7, A and C). A 10,700.0-Da fragment of TraR composed of residues 1–91, was present at similar intensities in digests of both free and complexed TraR (Fig. 7, A and C; data not shown). These results suggest first that free TraR has two sites sensitive to trypsin, Arg-91 and Lys-177 (Fig. 7, A and D), at which catalysis yields the 9217.0-Da fragment corresponding to residues 92–177 and, second, that the Lys-177 but not the Arg-91 site is masked by TraM in the TraR-TraM complex, leading to the accumulation of the 15,820-Da fragment corresponding to residues 92–234 and the decrease of the 9217.0-Da fragment corresponding to residues 92–177 in the digests of the TraR-TraM complex (Figs. 7, A and C).

As described under “Experimental Procedures,” the TraM protein used in this study has three extra amino acids, Gly-Ser-Glu, at the N terminus. Therefore, the molecular mass of the full sized GSH-TraM protein is 11,508 Da. Trypsin digestion of free GSH-TraM yielded a single polypeptide of 10,120.0 Da, corresponding to residues 11–102 (Fig. 7B), suggesting that Lys-10 is the only site on the TraM dimer sensitive to digestion by trypsin (Fig. 7E). Interestingly, the cleavage site at Lys-10 is

![Image](313x555 to 427x701)

**FIGURE 5.** TraMQ82A forms intermediate complexes with free TraR and DNA-bound TraR. Samples of purified free TraR alone (open triangle), and free TraR mixed with purified TraM (open circle) or mixed with purified TraMQ82A (filled circle) in a molar ratio of 1:2 were subjected to gel filtration chromatography (A). The DNA-bound TraR mixed with TraMQ82A (B) in a molar ratio of 1:2 also was analyzed by gel filtration chromatography as described under "Experimental Procedures." The fractions eluting at a position of 77 kDa containing TraMQ82A, TraR, and DNA from the experiment in B were combined and subjected to gel filtration chromatography following 30 min of incubation at 4 °C (C). To compare the elution profiles between experiments in B and C, we overlaid the chromatogram from C (solid line, labeled at the left y axis) with that from B (dotted line, labeled at the right y axis). The protein content and composition in the resulting chromatographic fractions was monitored by absorbance at 280 nm (mA280) and SDS-PAGE (data not shown). Peaks corresponding to the 151-kDa complex (TraM-TraR), the 77-kDa complex (TraM-TraR-DNA), TraR, TraM, and DNA are indicated in the figure. The dashed arrowhead in A indicates the new complex (69 kDa) formed by interactions between TraR and TraMQ82A.

Proteolytic Fingerprinting of TraR, TraM, and Their Complex—To probe the interaction interfaces on TraR and on TraM, we examined the trypsin sensitivity of the 151-kDa complex in comparison with those of the two individual proteins. Fifty-μl volumes of TraR, TraM, and the TraR-TraM complex at a concentration of about 1.5 mg/ml were digested with trypsin at a final concentration of 0.04 mg/ml for 1 min, 30 min, 1 h, 2 h, and overnight, respectively. SDS-PAGE analysis of these treated samples indicates that the digestion patterns are relatively stable between 30 min and 2 h of treatment (data not shown). Based on this information, we selected 1-h trypsin-digested samples for analysis by mass spectrometry to identify the polypeptide species occupying the protein bands observed on SDS-polyacrylamide gels.

Free TraR yielded as the most abundant species a fragment at 9217.0 Da corresponding to residues 92–177 of TraR. However, this fragment was barely detectable in the digests of the 151-kDa complex (Fig. 7, A and C). A second fragment at 15820.0 Da corresponding to residues 92–234 of TraR was dominant in the digests of the 151-kDa complex but was barely detectable in the digests of free TraR (Fig. 7, A and C). A 10,700.0-Da fragment of TraR composed of residues 1–91, was present at similar intensities in digests of both free and complexed TraR (Fig. 7, A and C; data not shown). These results suggest first that free TraR has two sites sensitive to trypsin, Arg-91 and Lys-177 (Fig. 7, A and D), at which catalysis yields the 9217.0-Da fragment corresponding to residues 92–177 and, second, that the Lys-177 but not the Arg-91 site is masked by TraM in the TraR-TraM complex, leading to the accumulation of the 15,820-Da fragment corresponding to residues 92–234 and the decrease of the 9217.0-Da fragment corresponding to residues 92–177 in the digests of the TraR-TraM complex (Figs. 7, A and C).

As described under “Experimental Procedures,” the TraM protein used in this study has three extra amino acids, Gly-Ser-His (GSH), at the N terminus. Therefore, the molecular mass of the full sized GSH-TraM protein is 11,508 Da. Trypsin digestion of free GSH-TraM yielded a single polypeptide of 10,120.0 Da, corresponding to residues 11–102 (Fig. 7B), suggesting that Lys-10 is the only site on the TraM dimer sensitive to digestion by trypsin (Fig. 7E). Interestingly, the cleavage site at Lys-10 is

![Image](313x555 to 427x701)

**FIGURE 6.** Interactions of TraM with dimeric TraR do not release 3-oxo-C8-HSL from the activator. Samples of purified active TraR (A, lane 2; B, lane 1), a mixture of TraR with TraM at a molar ratio of 1:1 (A, lane 3), and the 151-kDa stable TraM-TraR complex (B, lane 3) were dialyzed extensively and extracted by ethyl acetate as described under “Experimental Procedures.” The relative amounts of 3-oxo-C8-HSL in the resulting samples were determined by thin layer chromatography as described under “Experimental Procedures.” Lane 1 in A and lane 2 in B contain 50 and 20 fmol of authentic 3-oxo-C8-HSL (Sigma), respectively. The amounts of TraR in samples analyzed in A were twice those in B.

![Image](313x555 to 427x701)
significantly occluded by TraR in the TraR-TraM complex; digestion of the 151-kDa complex leads to the accumulation of intact GSH-TraM and a decrease in the amount of the 10,120.0-kDa fragment in the digests (Fig. 7, B and C; data not shown).

Residues of TraR Important for Interacting with TraM—Our previous mutational studies identified three residues of TraR, Pro-176, Leu-182, and Ala-195, that are important for interaction with TraM (12). However, these residues do not form a contiguous patch on the surface of TraR. We then tested a series of mutants of traR containing substitutions or single-residue deletions in the C-terminal region of the activator protein. All tested mutant TraR proteins are stable as assessed by Western analysis (Fig. 8A; data not shown). Of the 21 new mutants tested, five exhibited significantly reduced binding to TraM as assessed by Far Western analysis (Fig. 8B). One mutant, Δ177K, failed to bind TraM, whereas three mutants, L174A, G188A, and T190I, exhibited apparent binding affinities more than 16-fold reduced from that of wild-type TraR. In addition, we previously reported that the double mutant M213I/R215H showed a defect in TraM binding (12). The single substitution mutant R215A constructed in this study binds TraM as poorly as the double mutant M213I/R215H (Fig. 8B).

The remaining 16 substitution mutations in TraR (E178A/R183G, W184A, M189A, E193A, D196A, V197A, E198A, G199A, V200A, K201A, K201A/N203D, S204A, V205A, L209A, E211K, and A212V) had no detectable effect on TraM binding (data not shown).

DISCUSSION

Our gel filtration studies and amino acid analysis indicate that TraM and free TraR interact in solution to form a stable, high molecular weight 151-kDa complex composed of two dimers of the anti-activator and two dimers of the activator (Figs. 1 and 4 and Tables 1 and 3). Our conclusion concerning the nature of the TraM-TraR complex is consistent with that of Vannini et al. (16) and Chen et al. (18) but differs from that of Chen et al. (15), in which they proposed that the complex is composed of one monomer of TraM and one monomer of TraR.

When incubated together, purified TraR and TraM form several TraM-TraR complexes, including the stable 2TraR$_2$-2TraM$_2$ (Peak II), an unstable slightly larger complex (Peak I), and very large aggregates that elute at the void volume of the gel filtration column (Peak 0) (Fig. 1). Among these, the 2TraR$_2$-2TraM$_2$ is the only stable form and is the most abundant species (Figs. 1 and 4A). Moreover, this complex forms in vivo in A.
The Structural Nature of the Interaction Interfaces—Our previous studies clearly showed that TraM binds to the C-terminal region of the activator (12). TraR, like most members of the LuxR family, consists of distinct N- and C-terminal domains that are connected by a flexible linker (29, 30). The N-terminal region includes autoinducer-binding and dimerization domains, whereas the C-terminal region contains an eared helix-turn-helix DNA binding motif composed of \( \alpha \)-helices 11 and 12 (24, 28–32). Consistent with our previous genetic analyses (12), the eight amino acids at positions 174, 176, 177, 182, 188, 190, 195, and 215 in TraR, at which substitutions or a deletion result in mutants defective in binding to TraM, are exclusively positioned in the C terminus of the activator, spanning from \( \alpha \)-helix 10 to \( \alpha \)-helix 12 (Fig. 9C) (12). Leu-174, Pro-176, and Lys-177, which are located at the beginning of \( \alpha \)-helix 10 (Fig. 9C), form an elongated surface patch (Patch I) adjacent to a small patch (Patch II) formed by Arg-215 in \( \alpha \)-helix 12, the recognition helix (29, 30). The two patches, which are positioned in an L-shape in each protomer of TraR as viewed along the DNA axis from either end, form the most promising interaction interfaces for TraM (Fig. 9A).

Because of the asymmetry of the TraR dimer (29, 30), the locations of the TraM binding patches of one protomer differ from those of the other protomer.
Characteristics of TraM-TraR Interactions
Characteristics of TraM-TraR Interactions

In this model we required that the interaction interfaces between TraM and TraR take into account surface residues of each component identified by genetics and Far Western analysis as being important for the interaction. In contrast, a second model, also predicted as a 2 dimer-2 dimer complex, proposed that the DNA recognition helix, α-helix 12, of one TraR pro- tomer contacts each narrow groove of one TraM dimer (16).

This latter model suggests that TraM mimics the major groove of DNA to facilitate interaction with the DNA-binding domain of TraR. However, the model is not consistent with the results of our mutational analysis of TraR, trypsin sensitivity of TraR in the TraM-TraR complex, or the observation that TraM binds to DNA-bound TraR at a site other than recognition helix 12 to form the ∼77-kDa TraM₂-TraR₂-DNA complex. There are two apparent structural differences between the two models. First, in our model, the two TraR dimers are arranged face-to-face, and both Patches I and II make contacts with the activator (16). The results of our trypsin digest analysis support our model, where the cryptic site at Lys-10 of TraM is

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partially hidden inside the hole of the donut form (Figs. 7 and 9C). Second, the two TraR dimers in our model are arranged anti-parallel in a back-to-back manner with the recognition helix of each facing to the outside of the ring. The N-terminal domains of one TraR dimer are positioned above the ring of the complex, whereas those of the second dimer are located below the ring in the opposite direction (Fig. 9C). However, in the second model, the two TraR dimers are located face-to-face in a parallel manner, and residues, including Leu-174, Pro-176, and Lys-177, known to be required for TraM binding as assessed by mutational analysis and proteolytic fingerprinting, neither make contact with nor are occluded by the antiactivator.

Stepwise Disruption of TraR-DNA Complex by TraM—Except for the P176L and P176S mutants, all TraR mutants defective in TraM binding completely lose DNA binding activity (12, 17). This observation suggests that residues of TraR involved in TraM binding are partially occluded by the antiactivator.

Proteolytic fingerprinting, neither make contact with nor are occluded by the antiactivator.

Moreover, in our model, although one protomer of DNA-bound TraR-DNA complex and forms an unstable TraMQ82A-TraR-2TraM2 complex with concomitant release of DNA. This species could correspond to the 77-kDa complex seen in the gel mobility shift assays (Fig. 3). Second, gel filtration chromatography yields an intermediate nucleoprotein complex that is composed of a TraR dimer, a TraM dimer, and double strand tra box DNA (Fig. 4D). This species could correspond to the ternary complex as seen in the gel mobility shift assays. This ~77-kDa complex is unstable and converts to the stable 2TraM2-2TraR3 complex with concomitant release of DNA (Fig. 4, E–G). Third, the Q82A mutant of TraM binds to the TraR-DNA complex and forms an unstable TraMQ82A-TraR-dsDNA complex as assessed by gel filtration assays (Fig. 5). Moreover, in our model, although one protomer of DNA-bound TraR presents a compatible TraM binding site, because of the asymmetry of the TraR dimer, the N-terminal overhang of the second protomer partially occludes its C-terminal TraM binding domain (Fig. 9A). TraM then, can only access the one protomer of the DNA-bound TraR dimer, accounting for the appearance of the ~77-kDa TraM2-2TraR3-dsDNA complex (Fig. 10B). Following the release of DNA, two such complexes may then interact to form the stable 2TraM2-2TraR3 species (Fig. 10).

Inhibition of Ti plasmid conjugative transfer by TraM joins a growing list of prokaryotic regulatory systems involving an activator-antiactivator complex, including NifA-NifL regulation of nitrogen fixation in Azotobacter vinelandii (33) and Klebsiella pneumoniae (34), ComK-MecA-ClpC regulation of competence in Bacillus subtilis (35), GcvA-GcvR regulation of oxidative cleavage of glycine (36), and CRP-CytR regulation of many operons in E. coli (37). In the cases of NifA-NifL and ComK-MecA, the antiactivator binds to and titrates the activator, thus preventing DNA binding and transcriptional activation (33–35). However, in the cases of GcvA-GcvR and CRP-CytR, the antiactivator and the activator bind promoter DNA in tandem, and the interactions between the antiactivator and the activator at the promoter inhibit transcriptional activation (36, 37). In this regard, the mechanism of antiactivation by TraM resembles that of NifA-NifL and ComK-MecA. However, in contrast to these two systems in which the antiactivation complexes normally dissociate in response to activation signals (38, 39), the complex formed between TraR and TraM is extremely stable. This key difference suggests that the role of TraM in the biology of the Ti plasmid conjugative transfer system is to permanently inhibit the activity of TraR.

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