Tet(M)-Promoted Release of Tetracycline from Ribosomes Is GTP Dependent

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Tet(M) protein, which displays homology to elongation factor G (EF-G), interacts with the protein biosynthetic machinery to render this process resistant to tetracycline in vivo and in vitro. To clarify the basis of the resistance mechanism, the effects of Tet(M) on several reactions which occur during protein synthesis were examined. The mechanism of action of Tet(M) has been clarified by two observations. The protein relieves tetracycline inhibition of factor-dependent tRNA binding and dramatically reduces the affinity of ribosomes for tetracycline when GTP is present. This reduction in drug affinity appears to be due to a large increase in the rate of tetracycline dissociation. Addition of Tet(M) to ribosome-tetracycline complexes results in displacement of bound drug. And, while Tet(M) and EF-G GTPase activities are tetracycline resistant, the two proteins differ in their sensitivities to fusidic acid, with the latter activity inhibited by the drug. Furthermore, while Tet(M) protects translation from tetracycline inhibition in a defined system, it is unable to substitute for either EF-G or elongation factor Tu.

Antibiotic resistance mediated by Tet(M) (6) reverses the inhibitory effects of tetracyclines at the level of protein synthesis. Tet(M) (6) and, recently, Tet(O) (28) have been purified to homogeneity, and these proteins, along with Otr(A) (21), possess activities that reverse tetracycline inhibition of protein synthesis in vitro. On the basis of their extensive homology, several other tetracycline resistance determinants, TetB(P) (25), Tet(S) (8), and Tet(Q) (20), are also thought to mediate resistance by a similar mechanism. Proteins which act in this manner have been called ribosome protection proteins. This set of tetracycline resistance proteins all have homology with GTPases which participate in protein synthesis, especially elongation factor G (EF-G), with 25% sequence similarity overall between Tet(M) and EF-G (6). The most highly conserved motifs shared by tetracycline-resistant ribosome protection proteins are those regions involved in binding and hydrolysis of guanine nucleotides, the G domain.

Tet(M) protein has been purified by virtue of its ability to protect protein synthesis from tetracycline inhibition in vitro (6). Purified Tet(M) also possesses a GTPase activity similar to that of EF-G (6), with GTP hydrolysis associated with both proteins stimulated 20-fold by the presence of empty ribosomes (6, 30). This property distinguishes EF-G and Tet(M) from elongation factor Tu (EF-Tu), which catalyzes negligible GTP hydrolysis under these conditions (19). Additional evidence that the Tet(M) GTPase is important for the tetracycline resistance mechanism comes from work on Tet(O) (12). Substitutions for Asn within conserved motif 4 <u>N</u>KXD of the G domain of Tet(O) result in decreased tetracycline resistance. However, the function of the ribosome-dependent GTP hydrolysis by Tet(M) or Tet(O) in mediating tetracycline resistance has not been established.

EF-G promotes translocation of peptidyl-tRNA and deacylated tRNA from the A and P sites, respectively, to the P and E sites of the ribosome in a reaction which is tetracycline insensitive (23). Thus, Tet(M) probably does not replace EF-G in resistant cells even though the ribosome-dependent (-stim-

ulated) GTPase activities have similar properties. On the other hand, aminoacyl-tRNA is delivered to the ribosome in a complex with EF-Tu and GTP, a step known to be tetracycline sensitive (27). However, Tet(M) (72,576 Da) is considerably larger than EF-Tu (43,000 Da), and homology is confined to the G-domain motifs; in addition, Tet(M) is unable to rescue strains in which EF-Tu or EF-G is temperature sensitive (6). Taken together, it seems unlikely that Tet(M) is a functional homolog of either EF-Tu or EF-G.

Clarification of the mechanism by which Tet(M), and related ribosome protection proteins, relieves the inhibitory effects of tetracycline on protein synthesis requires detailed biochemical characterization of Tet(M)-associated activities during different steps of protein synthesis. In this paper, I demonstrate that Tet(M) displaces tetracycline from ribosome-drug complexes in a GTP-dependent manner. In view of the homology of Tet(M) to elongation factors, I have also compared biochemical activities of Tet(M) with those of EF-G and EF-Tu.

MATERIALS AND METHODS

Materials. Tet(M) was purified to apparent homogeneity by a slight modification of the method previously described (6) in which the Sephacryl 200 and hydroxylapatite steps were reversed and the Affi-Gel heparin column was eliminated. The final preparation was greater than >95% pure as judged by electrophoresis of 5 μg of Tet(M) through a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (16). The specific activity of this preparation was comparable to that described previously (6).

Ribosomes were prepared from *Escherichia coli* MRE600 (University of Alabama, Birmingham) as previously described (26). Ribosomes were suspended to an A_{260} of 1,400 in 20 mM Tris-HCl (pH 7.5)–20 mM MgCl$_2$–60 mM NH$_4$Cl–1 mM dithiothreitol and stored in aliquots at −75°C. EF-Tu was isolated from cells induced to express high levels of a glutathione *S*-transferase–EF-Tu fusion protein according to the method of Knudsen et al. (15). The final preparation of EF-Tu–GDP obtained after removal of the glutathione *S*-transferase moiety was judged to be >90% pure by denaturing gel electrophoresis. EF-G was purified from ribosome-free extracts (S150) of MRE600 cells by monitoring ribosome-dependent GTPase activity during fractionation through AGMP1 (2), hydroxylapatite, and Sephadex G-100. Phenylalanyl-tRNA synthetase was purified according to the method of Peterson and Uhlenbeck (22) from cells carrying the hybrid plasmid pB1 which contains the *pheS-pheT* locus (10). The final preparations of EF-G and phenylalanyl-tRNA synthetase were judged to be >90% pure by denaturing gel electrophoresis.

[³H]Phe-tRNA (∼4,000 cpm/pmol) was prepared by aminoacylation of tRNA^{Phe} in reaction mixtures containing 10 μM [³H]phenylalanine (8 Ci/mmol) and 3 μM tRNA^{Phe} in 1 ml of 100 mM Tris-HCl (pH 7.5)–50 mM NH$_4$Cl–20 mM MgCl$_2$–5 mM ATP for 30 min at 37°C. After the addition of 50 μl of 2 M

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potassium acetate (pH 5.0), the mixture was extracted twice with water-saturated phenol and concentrated by butanol extraction. Following precipitation with 2.5 volumes of ethanol overnight at −20°C, the mixture was centrifuged for 15 min at 4°C. The pellet was dissolved in 10 mM potassium acetate (pH 5.0), and charged tRNA^{Phe} was further purified by chromatography through Sephadex G-25 in the same buffer.

GTP hydrolysis. Tet(M) and EF-G were assayed for ribosome-dependent GTP hydrolytic activity as previously described (6) in reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 100 mM NH$_4$Cl, 10 mM magnesium acetate, 3 mM [γ-^{32}P]GTP, and 0.5 μM ribosomes and Tet(M), EF-G, and inhibitors as indicated. Reactions were initiated by the addition of GTP to a mixture of the other components which had been preincubated at 37°C for 3 min. Samples (0.05 ml) were withdrawn at timed intervals and pipetted into a slurry (0.45 ml) of activated charcoal (22% packed volume in 1 M HCl–0.1 M sodium PP$_i$–2 mg of bovine serum albumin per ml) to terminate the reaction. The charcoal was pelleted by centrifugation after 20 min of incubation on ice, and the ^{32}P in the supernatant was quantitated by liquid scintillation in Safety Solve (Research Products International Corp., Mt. Prospect, Ill.). Hydrolysis in the absence of either factor and ribosomes was subtracted from identical samples so that only the ribosome-dependent activity is reported.

A-site binding of tRNA. Binding of Phe-tRNA^{Phe} to the ribosome A site was performed by the three-step procedure of Cunningham et al. (9). First, the P site was filled with uncharged tRNA^{Phe} by incubating 187 nM 70S ribosomes with 2 μM uncharged tRNA^{Phe} in buffer A (50 mM Tris-HCl [pH 7.5], 75 mM NH$_4$Cl, 75 mM KCl, 5 mM dithiothreitol) with 10 mM Mg(OAc)$_2$ and 50 μg of poly(U) ml^{-1} for 10 min at 37°C. Ternary complexes were prepared by incubating 2 mM GTP with 3.75 μM EF-Tu in buffer A with 5 mM Mg(OAc)$_2$ for 15 min at 37°C prior to the addition of 1.0 μM [^{3}H]Phe-tRNA^{Phe} for an additional 5 min. For binding, 40 μl of ribosome mixture was mixed with 10 μl of ternary complex and incubated for 20 min. Reactions were stopped by the addition of 3 ml of ice-cold wash buffer [50 mM Tris (pH 7.5), 50 mM KCl, 20 mM Mg(OAc)$_2$], filtered through nitrocellulose filters (pore size, 0.45 μm), and washed twice with 3 ml of wash buffer. Background binding in the absence of EF-Tu was approximately 10% of that in its presence and was subtracted from comparable samples prepared in the presence of EF-Tu.

Polymerization assays. Reaction mixtures (0.05 ml) contained 500 nM 70S *E. coli* ribosomes, 250 μg of poly(U) ml^{-1}, 20 mM Tris-HCl (pH 7.5), 15 mM Mg(OAc)$_2$, 60 mM NH$_4$Cl, 7.5 mM phosphoenolpyruvate, 30 μg of pyruvate kinase ml^{-1}, 3 mM ATP, 1 mM GTP, 1 mM tRNA (bulk tRNA from *E. coli* MRE600–0.5 μM tRNA^{Phe}), 5 mM dithiothreitol, 45 nM EF-G, 225 nM EF-Tu, and 0.8 nM phenylalanyl-tRNA synthetase. After incubation at 37°C for 10 min, reactions were started by adding [^{3}H]phenylalanine (545 cpm/mol) to 20 μM. The concentrations of elongation factors (EF-G and EF-Tu) and phenylalanyl-tRNA synthetase were such that they were not limiting for synthesis. Reactions were terminated after 30 min by the addition of 3 ml of 10% trichloracetic acid and heated at 90°C for 15 min. After cooling, samples were passed through GF/C filters and washed with 10% trichloracetic acid–ethanol, and filters were counted after drying.

[^{3}H]tetracycline binding. [^{3}H]tetracycline (550 cpm/pmol) binding to 70S ribosomes was measured by combining [^{3}H]tetracycline as indicated in the text with ribosomes (final concentration of 500 nM) in a volume of 100 μl. Binding of [^{3}H]tetracycline was performed in 20 mM Tris (pH 7.5)–75 mM NH$_4$Cl–75 mM KCl–20 mM Mg(OAc)$_2$–5 mM dithiothreitol with 1 mM GTP present as described in the text. For the reactions at 2°C, mixtures were incubated first at 37°C for 10 min, [^{3}H]tetracycline was added for 10 min, and reaction mixtures were then cooled to 2°C for 5 min. Unlabelled tetracycline, EF-G, or Tet(M) was added, and samples were withdrawn, immediately filtered, and washed. [^{3}H]tetracycline binding was estimated from the radioactivity recovered after samples were filtered through 0.45-μm-pore-size nitrocellulose filters and washed twice with 3 ml of ice-cold wash buffer [50 mM Tris (pH 7.5), 50 mM KCl, 20 mM Mg(OAc)$_2$]. Background levels of radioactivity obtained from reactions in the absence of ribosomes were subtracted from experimental values.

Antibody purification and quantitative Western blot (immunoblot). Affinity-purified anti-Tet(M) antibody [AP-anti-Tet(M)] was isolated from polyclonal sera prepared against Tet(M) in New Zealand White rabbits by standard procedures (1). Quantitation of Tet(M) in cells was determined by immunoblotting (7). Briefly, samples containing known numbers of cells were compared with samples containing known amounts of purified Tet(M) protein; following separation of proteins by denaturing gel electrophoresis (16) and transfer of the proteins to membranes (29), the presence of Tet(M) was detected by chemiluminescence (Protein Images; U.S. Biochemical) with AP-anti-Tet(M) antibodies.

Chemicals and enzymes. Poly(U), pyruvate kinase, tRNA^{Phe}, and Sephadex G-25 were purchased from Sigma (St. Louis, Mo.). Phosphoenolpyruvate and bulk *E. coli* tRNA were from Boehringer Mannheim (Indianapolis, Ind.), while ATP and GTP were from Pharmacia (Piscataway, N.J.). L-[ring-2,6-^{3}H(N)]phenylalanine (60 Ci/mmol) and 5′ [γ-^{32}P]GTP (5,000 Ci/mmol) were obtained from Amersham (Arlington Heights, Ill.), and [7-^{3}H(N)]tetracycline (0.6 Ci/mmol) was obtained from NEN DuPont (Wilmington, Del.). *E. coli* MRE600 cell paste for preparation of ribosomes and EF-G were purchased from the University of Alabama, Birmingham.
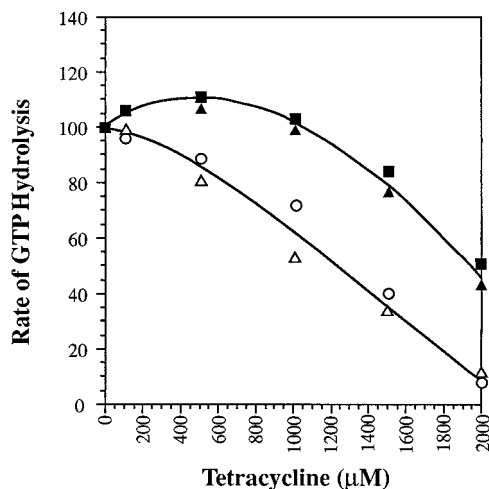

FIG. 1. Tetracycline sensitivity of the GTPase associated with Tet(M) and EF-G. The rate of ribosome-dependent GTP hydrolysis was measured as described in Materials and Methods in reaction mixtures containing from 0 to 2,000 μM tetracycline. The relative rate of 100 represents an absolute rate of ~60 mol of GTP hydrolyzed per min per mol of EF-G (△ and ○) or Tet(M) (▲ and ■) in the absence of tetracycline. Different symbols represent separate experiments.

RESULTS

Tet(M)-associated GTPase is fusidic acid resistant in vitro. Tet(M) protein shows striking similarity to EF-G at the amino acid sequence level, and both proteins possess ribosome-dependent GTPase activities that are independent of ongoing translation (6). This uncoupled GTPase activity requires only the presence of ribosomes and EF-G or Tet(M) (6). In the experiments described here, GTP hydrolysis was linear with respect to both time and protein concentrations at a rate of 60 mol/min/mol of EF-G or Tet(M). The GTPase activity of the protein [EF-G or Tet(M)] was stimulated 18- to 20-fold by ribosomes.

Since Tet(M) mediates tetracycline resistance, the tetracycline sensitivities of the GTPase activities of these two factors were compared even though tetracycline does not inhibit the ability of EF-G to hydrolyze GTP (13). As can be seen in Fig. 1, tetracycline affects EF-G or Tet(M) only at high concentrations. Even though the Tet(M) GTPase is about twofold more resistant to tetracycline, retaining 50% activity at 2 mM tetracycline compared with 1 mM for EF-G, this difference probably does not account for the tetracycline resistance activity of Tet(M) during translation. These concentrations of tetracycline are more than 10-fold greater than the intracellular concentration necessary to inhibit protein synthesis (4). One interesting feature of the tetracycline inhibition of the GTPase is that the Tet(M)-mediated reaction is slightly stimulated (10% increase) by up to 1 mM tetracycline.

Fusidic acid inhibits the EF-G-mediated GTPase by binding to the EF-G ribosome complex, stabilizing EF-G–GDP on the ribosome after one round of hydrolysis (31), thus preventing further hydrolysis. When the fusidic acid sensitivity of the Tet(M)-associated GTPase was compared with that of EF-G, the Tet(M)-mediated activity was much more resistant to fusidic acid than was that of EF-G (Fig. 2). The EF-G activity was inhibited 50% by 2 μM drug while comparable inhibition of GTP hydrolysis by Tet(M) was observed only at 800 μM fusidic acid.

Tet(M) protects factor-dependent tRNA binding from inhibition. The favored mechanism of action of tetracycline is
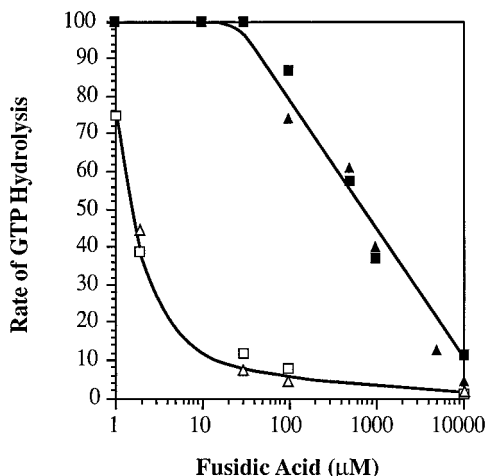
FIG. 2. Fusidic acid sensitivity of the ribosome-dependent GTPase associated with Tet(M) and EF-G. The rate of ribosome-dependent GTP hydrolysis by Tet(M) (▲ and ■) and EF-G (△ and □) was measured in the presence of fusidic acid from 1 to 10,000 μM compared with the rate of hydrolysis (60 pmol per min per pmol of protein) observed in the absence of drug, taken as 100. Different symbols represent separate experiments.

inhibition of binding of aminoacyl-tRNA to the A site of codon-programmed ribosomes. The effect of Tet(M) on EF-Tu-mediated binding of aminoacyl-tRNA to the A site was therefore examined. At concentrations of 1 to 50 μM tetracycline, binding of aminoacyl-tRNA is inhibited to a significant degree. However, in the presence of Tet(M) protein, binding of aminoacyl-tRNA to ribosomes is not significantly affected by tetracycline at these concentrations. Even at 500 μM tetracycline, binding in the presence of 1.5 molar excess of Tet(M) over ribosomes is nearly 75% of that found in the absence of tetracycline (Fig. 3).

The extent of protection is dependent on the amount of Tet(M) present in the reaction mixtures as well as the concentration of tetracycline present (Fig. 4). While Tet(M) protein has no effect on binding in the absence of antibiotic, it does relieve tetracycline inhibition even at ratios of less than 1 mol of Tet(M) per mol of ribosomes. However, protection is not complete even at 1.7 Tet(M) molecules per ribosome; under
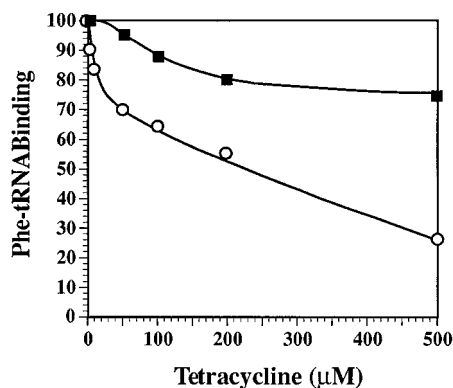

FIG. 3. Tetracycline sensitivity of Phe-tRNA binding. Phe-tRNA binding (shown as percentage of binding in the absence of tetracycline) to ribosomes was measured as described in Materials and Methods in the presence (■) or absence (○) of 1.5 mol of Tet(M) per ribosome at varying concentrations of tetracycline. Blanks, in the absence of EF-Tu, were subtracted. 70S ribosomes bound 0.65 mol of Phe-tRNA per ribosome in the presence or absence of Tet(M).
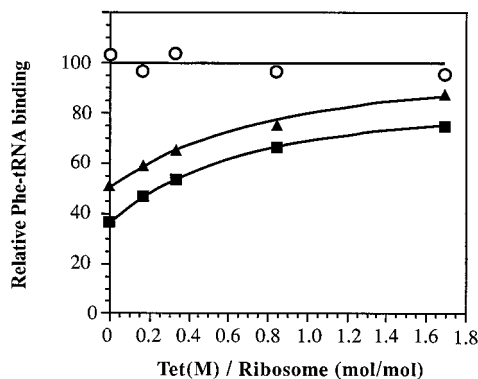

FIG. 4. Phe-tRNA binding at varying concentrations of Tet(M). Phe-tRNA binding to ribosomes was measured as described in Materials and Methods at varying ratios of Tet(M) to ribosome in reaction mixtures containing tetracycline at 0 (○), 200 (▲), or 500 (■) μM. Ribosomes were present at a 150 nM final concentration. Blanks, in the absence of EF-Tu, were subtracted.

these conditions, binding of Phe-tRNA^{Phe} is restored to 75% at 500 μM tetracycline and to 90% at 200 μM tetracycline. No Phe-tRNA^{Phe} binding to ribosomes was found when Tet(M) replaced EF-Tu in these reactions, indicating that Tet(M) is unable to promote factor-dependent tRNA binding by itself.

Immunoblotting with affinity-purified rabbit anti-Tet(M) antibodies [AP-anti-Tet(M)] was used to estimate the level of Tet(M) protein present in *E. coli* strains expressing different levels of tetracycline resistance. In data not shown, samples of cultures corresponding to a known number of cells were electrophoresed in the same gel with known amounts of Tet(M) and the gel was subjected to immunoblot analysis with AP-anti-Tet(M). By this method, cells expressing resistance to 6.25 μg of tetracycline per ml were estimated to contain 1,300 molecules of Tet(M) per cell while cells expressing resistance to 100 μg of antibiotic per ml were estimated to contain 8,500 molecules of Tet(M) per cell. When correction was made for growth rate and probable cell size, the concentrations were calculated to be 1.7 μM (cells resistant to 6.25 μg of tetracycline per ml) and 17.3 μM (cells resistant to 100 μg of drug per ml). The number of ribosomes present in rapidly growing *E. coli* is estimated to be ~6,500 to 15,000 per cell (11 to 25 μM) (3). Therefore, Tet(M) is present in the range of 0.1 to 1 mol per mol of ribosomes, similar to the molar ratios shown in Fig. 4.

Polyphenylalanine synthesis is protected by Tet(M). Tet(M) is purified by monitoring its ability to confer tetracycline resistance on protein synthesis reactions with ribosomes and crude enzyme fraction (S150) from drug-sensitive cells (6). A similar protection was also observed in defined protein synthesis reactions in which purified elongation factors (EF-G and EF-Tu) and phenylalanine tRNA synthetase are used in place of the S150 fraction (Fig. 5). In the absence of Tet(M), synthesis is inhibited significantly at both 200 and 500 μM. Addition of 0.4 mol of Tet(M) per ribosome completely reversed the inhibitory effects of 200 μM tetracycline while 0.4 mol of Tet(M) per ribosome gave 85% protection in 500 μM drug. Under these conditions, Tet(M) has no effect on the rate of protein synthesis in the absence of antibiotic. In experiments not shown, tetracycline has been shown to affect the rate of synthesis and Tet(M) restored the rate to that observed in the absence of drug. The presence of high concentrations of Tet(M) inhibited synthesis even in the absence of antibiotic (Fig. 5, open symbols) while heat-treated (75°C for 15 min) Tet(M) was not inhibitory. Although the basis of this inhibition is not clear,
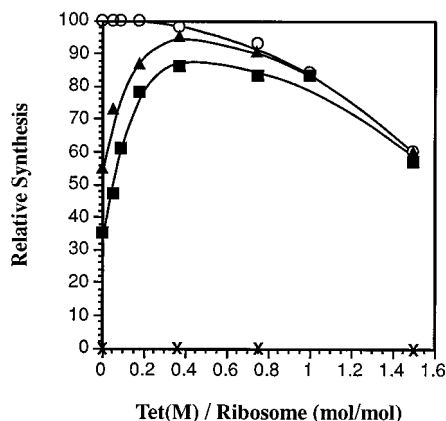
FIG. 5. Tet(M) relieves tetracycline inhibition during protein synthesis. Poly-phenylalanine synthesis [shown as percentage of synthesis in the absence of drug and Tet(M)] was measured as described in Materials and Methods at 0 (○), 200 (▲), and 500 (■) μM tetracycline as a function of the ratio of Tet(M) to ribosomes present in the reaction mixtures and in reaction mixtures containing Tet(M) in place of either EF-G or EF-Tu (✖). Ribosomes were present at 500 nM, and Tet(M) was present at 0 to 750 nM [0 to 1.5 mol of Tet(M) per ribosome]. Synthesis in the absence of drug and Tet(M) in these reactions, 250 pmol of phenylalanine incorporated, was taken as 100%.

10-fold elevation of the amount of EF-G or EF-Tu did not relieve this inhibition. Furthermore, no synthesis occurred if reaction mixtures contained Tet(M) in place of either EF-G or EF-Tu (Fig. 5), showing that Tet(M) was unable to substitute for either elongation factor.

Tet(M) releases tetracycline bound to ribosomes in a GTP-dependent reaction. It has been suggested that ribosome protection proteins such as Tet(M) and Tet(O) facilitate the binding of aminoacyl tRNA to the ribosome even in the presence of bound antibiotic (12, 17). Since Tet(M) has been shown here to protect tRNA binding to the ribosomal A site and protect protein synthesis from tetracycline inhibition, it would follow that, if tetracycline were to remain bound, the binding site must be altered. To begin studies on the nature of this altered binding, the affinity of ribosomes for [³H]tetracycline was determined in the presence and absence of purified Tet(M) protein. In both instances, ribosome affinity for the drug was similar, with an equilibrium dissociation constant (K_d) of 5 μM, a value in reasonable agreement with previous determinations by fluorescent anisotropy methods (11). However, if GTP was added to the reaction mixtures, a different result was obtained. The association of tetracycline with ribosomes was unaltered in the absence of Tet(M) protein but greatly reduced ($K_d = 55$ μM) in the presence of Tet(M) (Fig. 6A). Although not shown, ribosomes engaged in protein synthesis also showed a similar decrease in their affinity for tetracycline upon the addition of Tet(M). Inclusion of EF-G and/or EF-Tu had no effect on tetracycline binding in the absence of Tet(M) protein. It is noteworthy that the concentration of available ribosome sites for tetracycline binding was not significantly different in the presence or absence of Tet(M) and GTP (0.57 and 0.45 μM, respectively) (Fig. 6A).

In order to test whether Tet(M) might displace tetracycline already bound to ribosomes, [³H]tetracycline was bound to ribosomes at 37°C in the presence of GTP and challenged at 2°C with either a 100-fold excess of unlabelled tetracycline or EF-G or Tet(M) to the same concentration as ribosomes. The results (Fig. 6B) show that bound tetracycline is not readily exchanged upon the addition of a 100-fold excess of unlabelled tetracycline, with the half-life at 2°C of greater than 20 min.
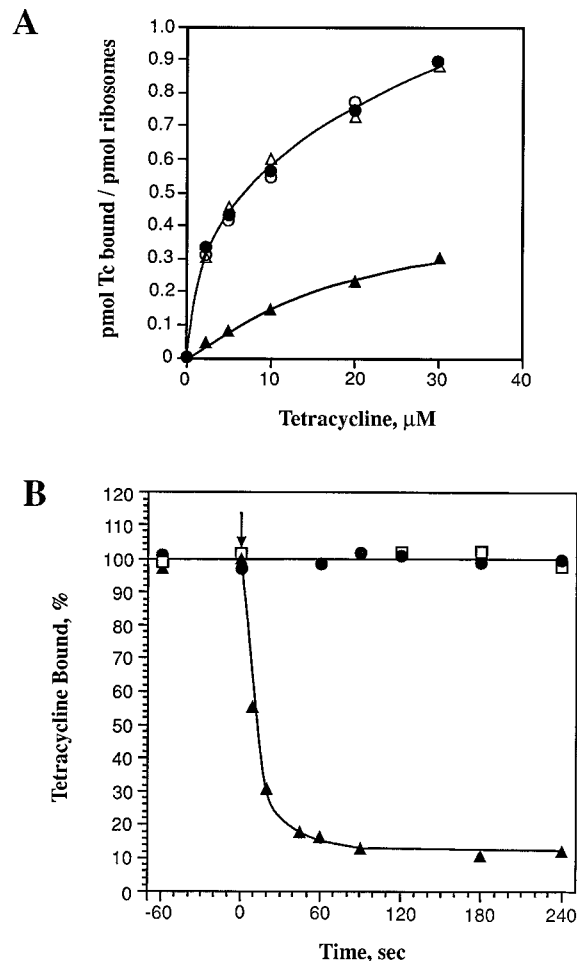

FIG. 6. GTP-dependent release of [³H]tetracycline from ribosomes by Tet(M). (A) Binding of [³H]tetracycline to ribosomes (● and ○) or ribosomes plus Tet(M) (▲ and △) was measured as described in Materials and Methods. Closed and open symbols indicate the presence or absence of GTP, respectively. Curves shown were obtained by Marquardt nonlinear least squares fit to a hyperbola (18) as were the values for the concentration of available tetracycline binding sites. (B) Binding of [³H]tetracycline to ribosomes in the presence of GTP was measured following a challenge with a 100-fold excess (500 μM) of unlabelled tetracycline (●), a molar equivalent of EF-G (□), or a molar equivalent of Tet(M) (▲) added at the time indicated by the arrow.

Similarly, EF-G had no effect on the release of tetracycline bound to ribosomes. However, tetracycline was readily released from ribosomes upon the addition of Tet(M) with a dissociation half-life at 2°C estimated to be about 10 s. At 37°C, the half-life for dissociation of tetracycline from ribosomes at 37°C was about 70 s, and in the presence of Tet(M), dissociation was complete by 10 s, indicating a half-life of less than 5 s. In experiments not shown, there is no evidence for tetracycline binding to Tet(M) alone.

Release of [³H]tetracycline from ribosome-Tet(M) complexes was also observed upon GTP addition. However, addition of 5′ guanylylimidodiphosphate, a nonhydrolyzable analog of GTP, resulted in only limited release (20% compared with GTP) of [³H]tetracycline, suggesting that hydrolysis may be required for full release of antibiotic (not shown). The small amount of release seen may be due to contamination of the 5′ guanylylimidodiphosphate with minor amounts of GTP.

DISCUSSION

The Tet(M) protein interacts with the bacterial translation system to render protein synthesis resistant to the presence of tetracycline, and although Tet(M) is available in pure form (6), the biochemical basis of resistance has been obscure. Various models have been proposed for Tet(M) action. One unlikely possibility is that Tet(M) is able to substitute for EF-G during protein synthesis. Tet(M) protein closely resembles EF-G; Tet(M) and EF-G are similar in molecular weight, they have amino acid sequence homology especially in the amino-terminal G domain, both associate with ribosomes in a salt-labile manner, and both stimulated ribosome-dependent GTP hydrolysis (5, 6). I have shown here that Tet(M) is unable to support polyphenylalanine synthesis with purified components in reactions in which Tet(M) is used in place of either EF-G or EF-Tu, thus indicating that Tet(M) cannot function as an elongation factor homolog, at least in *E. coli*.

Tetracycline is known to inhibit the binding of aminoacyl tRNA to ribosomes. The experiments presented here clearly show that EF-Tu-dependent tRNA binding to ribosomes is protected from tetracycline inhibition when Tet(M) is present. However, Tet(M) cannot substitute for EF-Tu in factor-dependent tRNA binding. Tet(M) also relieves tetracycline inhibition of polyphenylalanine synthesis in factor-dependent translation reactions. While Tet(M) is unable to substitute for EF-G under these conditions, it does inhibit protein synthesis to a significant degree, perhaps because the binding sites for these two proteins overlap on the ribosome.

The ribosome-dependent GTPase and ribosome translocation activities of EF-G are not inhibited by tetracycline (23). In this context, I have shown here that the ribosome-dependent GTPase activities of both EF-G and Tet(M) are tetracycline resistant to approximately the same extent and that GTPase activity associated with Tet(M) is fusidic acid resistant in vitro. This fusidic acid resistance is not surprising given that there are several amino acids in the Tet(M) sequence which correspond to amino acids found in fusidic acid-resistant mutants of EF-G (14). In vivo, cells expressing Tet(M) do not show increased resistance to fusidic acid (6a). Although this finding might also suggest that Tet(M) is unable to replace EF-G in vivo, it is known that fusidic acid sensitivity is dominant in this system. Furthermore, I have found that Tet(M) fails to complement temperature-sensitive EF-G mutations in vivo (reference 6 and unpublished data).

It has been reported elsewhere (12, 17) that Tet(O), a ribosome protection protein with 75% sequence similarity to Tet(M), does not interfere with tetracycline binding to ribosomes. These authors propose that ribosome protection proteins [Tet(M), Tet(O), etc.] allow entry of aminoacyl-tRNA to the ribosome A site even when tetracycline is bound at this site (12, 17). These experiments were carried out with crude ribosomes isolated from resistant and sensitive cells, and it was not shown that the resistance protein was associated with ribosomes. In contrast to this, I have shown here that Tet(M) promotes the release of tetracycline from ribosomes in a reaction that is dependent on the presence of GTP.

It is feasible that in vivo Tet(M) acts in conjunction with EF-G as part of a translocation complex at a step just prior to A-site binding of aminoacyl-tRNA to release any antibiotic bound near this site. According to the allosteric three-site model of Rheinberger and Nierhaus (24), ribosomes in the posttranslocation state, immediately subsequent to EF-G action, have P sites and E sites in high-affinity states for tRNA binding and with an A site in a low-affinity state. The ribosome is ready for EF-Tu to bring tRNA to the A site, and it is binding of the correct tRNA which induces the pretranslocation conformational change. If tetracycline were to bind to the A site, tRNA binding would be inhibited. Thus, the posttranslocation conformation might be the context for Tet(M) action. Experiments to define the Tet(M) binding site on pretranslocation and posttranslocation ribosomes will help clarify when and where Tet(M) acts to keep the A site free of bound tetracycline.

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