The NH$_2$-terminal Half of the Tn10-specified Tetracycline Efflux Protein TetA Contains a Dimerization Domain*

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The 43.1-kDa tetracycline-cation/proton antiporter TetA from Tn10 comprises two equal-sized domains, α and β (amino-terminal and carboxyl-terminal halves, respectively). An inactivating mutation in the α domain can complement a mutation on a second polypeptide in the β domain to restore partial tetracycline resistance in bacterial cells, suggesting that intermolecular interactions permit this transport protein to act as a multimer. In the present studies, multimer formation was examined in mixtures of dodecylmaltoside extracts of membranes from Escherichia coli containing different TetA derivatives. TetA, TetA$_{αβ}$, and TetA$_{βα}$ were each fused genetically to a six-histidine carboxyl-terminal tail. The ability of these fusions, immobilized on a nickel affinity column, to bind wild-type TetA or other Tet fusions was determined. An interaction between α domains on different polypeptides which resulted in multimerization was seen. The binding was specific for Tet protein and did not occur with other membrane proteins or another polyhistidine fusion protein. No α-β interactions were detected by this method, although they are postulated to occur in the intact cell based on the α-β genetic complementations. A dimeric model for TetA having intermolecular α-α and α-β interactions is presented.

TetA(B), a cytoplasmic membrane protein encoded by Tn10, is a member of a family of related tetracycline efflux proteins in Gram-negative bacterial cells (1, 2). It mediates resistance to tetracyclines by pumping a cation-tetracycline complex across the membrane outwardly in an electroneutral exchange for an inwardly moving proton (3–6). Experiments with a collection of point mutations have shown that inactivating mutations in the first half of the protein complement those in the second half in cells containing both polypeptides (7, 8). Complementation also occurred with protein fragments (9). However, each half of TetA did not have a unique function completely independent of that of the other half, since full or even half-resistance was rarely restored in complementations, even in cases where the presence of both polypeptides was confirmed. These results suggested that synergistic physical interaction between the two halves of the protein was required for resistance, and that such interaction could occur intermolecularly in a dimeric or higher multimeric state.

Further evidence for the required interaction between the two halves and for dimerization came from Tet protein chimeras. The sequences of the related tetA genes from the family of tetracycline resistance determinants predicts that each TetA protein has two sets of six putative membrane-spanning α-helices separated by a putative large cytoplasmic loop (2, 9–12). TetA proteins from classes A and C are more closely related (76%) than either are to the class B (Tn10) protein (45%) (1). An “A/C” chimera, containing the first (α) half from class A and the second (β) half from class C, was active in expressing tetracycline resistance, whereas a B/C or C/B chimera was not (13). Evidently the α and β halves functioned together only if they were related closely enough. The B/C and C/B chimeras together in the same cell, however, showed about 20% complementation of tetracycline resistance, indicating multimer formation (13). α-β interaction was also suggested by the ability of the cloned α half to stabilize the cloned β half when both were present on separate polypeptides in the same cell (14). Complementation occurred in this case also.

The present work was designed to determine whether TetA extracted from the cell existed as a multimer. We genetically fused six histidines to the carboxyl terminus of TetA, TetA$_{α}$, and TetA$_{β}$ of class B. The ability of such a “6H” fusion to bind different Tet protein molecules was measured using Ni$^{2+}$ affinity chromatography.

**EXPERIMENTAL PROCEDURES**

Construction and Description of Plasmids—See Table I for summary of plasmids and Fig. 1 for diagrams of protein constructs.

pACT7 (encoding T7 RNA polymerase regulated by the lacUV5 promoter) (Kan, p15A origin). This plasmid (15) was used in trans with pET21b-Tet6, pLY17, and pLY22.

pET21b-Tet6 (encoding Tet-6H, 45.6 kDa). This plasmid, derived from pET21b (Novagen), provides a T7 promoter and lac operator regulating Tet-6H. It also provides lac, the gene for the lac repressor, and was used in conjunction with pACT7. Its construction has been described. Tet-6H comprises an initial methionine followed by (in order) an 11-residue “T7 tag,” TetA (minus the initial methionine), leucine, glutamate, and then the six histidines (6H).

pLY17 (encoding Tetα-6H, 24 kDa). A 0.6-kilobase EcoRI-XhoI fragment representing the β half of TetA was deleted from pET21b-Tet6. The 5’ ends were filled in with Klenow DNA polymerase prior to ligation. TetA$_{α}$ was thereby in frame with the polyhistidine tail encoded 3’ to the XhoI site. Loss of the 0.6-kilobase fragment was confirmed by loss of the Scal site within it, and by the 6.0 kilobase size of the resulting plasmid. pLY17 was used in combination with pACT7.

pLY22 (encoding Tetβ-6H, 24 kDa). The same tacPCR product used to make pET21b-Tet6 was restricted with EcoRI (in the central loop of TetA) and cloned into identically restricted pET21b. This put the TetA$_{β}$ domain in-frame with both the upstream “T7 tag” and the downstream polyhistidine tail encoded by pET21b. pLY22 was used in combination with pACT7.

pMalc-Tet1 (encoding Macl-Tet, 86 kDa). A tetA PCR product having BamHI sites on each end was restricted with BamHI and cloned into BamHI-restricted pMAL-C2 (New England Biolabs). This created an in-frame fusion between maltose-binding protein MalE (missing its
signal sequence) and the (cytoplasmic) amino terminus of the intact fusion protein, the largest and most abundant migrating at 70 kDa. The fusion protein was cleavable between MalE and TetA by factor Xa, as described (9). The TetA fusion gene was regulated by the tetB-6H gene and having a pMB1 origin of replication. A second mutant plasmid known to encode an active class B TetA domain and which had the compatible p15A origin of replication and encoded CmR was also introduced; the second plasmid was either pLR1097 (bearing wild type tetA with a deletion in the β-domain (9)) or pRAR1032 (bearing the B/C chimeric gene (13)). In these second plasmids the mutant tetA gene was regulated by the tet repressor TetR; non-inhibitory autoinduced chlorotetacycline (10 μg/ml) was used for induction (7). Resistance to tetracycline was measured by gradient plates (7) containing the autoinduced chlorotetacycline and 20 μM IPTG.

Preparation of Membrane Extracts, Use of Ni-NTA Columns, and Subsequent Analyses—Growing cells bearing the appropriate plasmids were induced (at A530 = 0.8) for 1.5–3 h with the appropriate agents (100 μM IPTG for pET21b-Tet6, plY17, plY22, pMalC-Tet1; 42 °C for pKRKH21; tetracycline at 2 μg/ml for R222; 0.02 μg/ml anhydrotetracycline (another gratuitous tet inducer (20)) for pRAR plasmids). Cells were harvested and used immediately or stored at –80 °C. Cells were lysed by sonication at A530 = 100 in 50 mM sodium phosphate, 2 mM MgCl2, 100 μM lysosome, pH 7.4. Membranes were sedimented (105,000 × g, 35 min, TLA 100.3 rotor (Beckman)) and resuspended in 10 mM sodium phosphate, pH 7.2 (0.3 ml/orignal 100 A350 units). n-Dodecyl-β-D-maltoside (Anatrace, 8%) was added to 1.5%. After 30 min of occasional mixing (4°C), unsolubilized membranes were sedimented as before and discarded. The detergent extracts were stored at –80 °C.

Two different dodecylmalto side extracts (usually 10–50 μl of each) were combined if desired to allow “mixed multimers” to form. After 30 min of occasional mixing at 4 °C, 1/7 volume of 8-fold concentrated column buffer was added (column buffer final concentration was 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5 mM imidazole, 0.03% dodecyl maltoside). Small (0.1 ml) columns of Ni-NTA (Qiagen) (Ni2+ bound to nitrilotriacetate immobilized on Sepharose CL-6B), were prepared in Pasteur pipettes and washed in column buffer. The samples were loaded onto the columns (50 μl every 5–7 min) and washed with column buffer (0.2 ml, 2 min, ×6). When desired, an elution in column buffer at 2 μg/ml for R222 (FII) (1, 18). It is compatible with both ori PMB1 and ori P15A plasmids. Expression of TetA was induced by tetracycline.
40 mM imidazole was then performed at the same rate. Finally, an elution in column buffer at 1 M imidazole (pH adjusted to 8) was done and eluted with 1 M imidazole, and both the original extracts and extractsof membranes from all cells were prepared. In one was also induced with tetracycline. Dodecylmaltoside deter-

tating the gene on Tn ring plasmid R222), or (iii) pET21b-Tet6 plus R222, were in-
duced with IPTG. The wild type gene in the strains with Tn tet-6H by immunodot blot using antisera and 125I-Protein A. The bound proteins were examined for wild type TetA and Tet-6H premixed before application (7). Blots were probed with antiCt (A) or antiTet antisemur (B).

**RESULTS**

Formation of Mixed Multimers between Wild Type TetA and Tet-6H—DH5α cells containing pACT7 plus (i) the vector containing the tet-6H gene (pET21b-Tet6), (ii) the vector (pET21b) alone plus the wild type tetA gene (pTn50f on naturally occurring plasmid R222), or (iii) pET21b-Tet6 plus R222, were in-
duced with IPTG. The wild type gene in the strains with Tn10 was also induced with tetracycline. Dodecylmaltoside detergent extracts of membranes from all cells were prepared. In one case, extracts from i and ii were mixed in equal volumes for 5 min. The extracts were passed through a Ni-NTA column to bind Tet-6H and co-associated proteins. Bound proteins were eluted with 1 M imidazole, and both the original extracts and the bound proteins were examined for wild type TetA and Tet-6H by immunodot blot using antisera and 125I-Protein A.

The original extracts loaded onto the Ni-NTA columns were then analyzed first. Probing with antiCt antisemur, which reacts with TetA but not with Tet-6H, revealed that the amount of TetA in the two strains bearing Tn10 was similar (Fig. 2, column A, rows 1 and 3). As expected, the extract from the strain with only Tet-6H (without Tn10) showed a low, probably host cell background, reaction with antiCt (Fig. 2, column A, row 2). Use of antiTet antisemur, which reacts similarly with both TetA and Tet-6H, showed that cells synthesized less wild type TetA than overproduced Tet-6H (Fig. 2, column B, row 1 versus row 2), as shown before (5).

The samples which bound to the Ni-NTA columns were then analyzed. Use of antiCt demonstrated that little wild type TetA bound to Ni-NTA in the absence of Tet-6H (Fig. 2, column A, row 4). On the other hand, in the presence of Tet-6H bound to the resin, TetA binding was clearly detectable. This was true whether the two versions of Tet had been synthesized in the same cell (Fig. 2, column A, row 6), or came from separate cells but were mixed together prior to loading onto Ni-NTA (Fig. 2, column A, row 7). If Tet-6H was loaded first, followed by TetA, TetA was still bound (Fig. 2, column A, row 5). Use of antiTet confirmed that similar amounts of Tet-6H were bound to Ni-NTA in all cases (Fig. 2, column B, rows 5–7). From Coomassie

**TABLE II**

Association of Tet279-LacZ with Tet-6H or 6H-IICBgc bound to Ni-NTA

| 6H fusion | pmol bound |
|-----------|------------|
| 1) None (host extract) | 0 | 2.3 | 0 |
| 2) 6H-IICBgc | 350 | 3.5 | 1.2 |
| 3) Tet-6H | 120 | 14.5 | 12.2 |

*After subtraction of host value in row 1.*

Experiments were performed in triplicate in 9 or 10% SDS-PAGE gels (21), and processed by SDS-PAGE sample buffer (21), and processed by SDS-PAGE (9 or 10%) 0.75-mm thick minigels. Some gels were then electroblotted onto Immobilon P (Millipore) and probed with antiCt or antiTet antisemur, followed by 125I-Protein A, as described. 1 AntiCt was specific for the carboxyl-terminal 14 amino acids of TetA (22); its reaction with TetA binding was clearly detectable. This was true whether the two versions of Tet had been synthesized in the same cell (Fig. 2, column A, row 6), or came from separate cells but were mixed together prior to loading onto Ni-NTA (Fig. 2, column A, row 7). If Tet-6H was loaded first, followed by TetA, TetA was still bound (Fig. 2, column A, row 5). Use of antiTet confirmed that similar amounts of Tet-6H were bound to Ni-NTA in all cases (Fig. 2, column B, rows 5–7). From Coomassie

**FIG. 2. Detection of wild type TetA and Tet-6H before and after Ni-NTA chromatography of dodecylmaltoside membrane extracts (dot-blot).** Rows 1–3, extracts applied to Ni-NTA (from 0.1 A530 units of cells): wild type TetA (1), Tet-6H (2), or both (3) from the same cell. Rows 4–7, 1 M imidazole eluates from Ni-NTA (from 0.5 A530 units of cells): wild type TetA (4), wild type TetA applied after Tet-6H (5), wild type TetA and Tet-6H from the same cell (6), or wild type TetA plus Tet-6H premixed before application (7). Blots were probed with antiCt (A) or antiTet antisemur (B).
loops of the β region.

β-β Interactions Were Not Required for Multimer Formation—In TetA-6H the β domain is completely absent. If indeed β-β interaction was not required for Tet-Tet binding, TetA-6H should be able to bind a Tet protein containing both α and β domains. As its prospective partner, we used the MalE-Tet fusion in which the entire TetA protein was fused genetically to MalE. The MalE-Tet polypeptide was not bound in absence of polyhistidine fusion (Fig. 3, lanes 1) nor to 6H-IICB9c (Fig. 3, lanes 2). MalE-Tet was, however, bound to TetA-6H (Fig. 3, lanes 3). These results extended the previous finding with Tet279-LacZ, showing that an α domain of TetA was sufficient for binding to full-length Tet.

Therefore, β-β interactions were not necessary. α-β Interactions Did Not Contribute to Multimer Formation—The genetic complementation which occurred between α and β domains on different polypeptides (7–9, 13, 14) had suggested that these two domains could interact physically. We looked for such an α-β interaction with TetA-6H. We mixed extracts containing either B/B (that is, wild type TetA from the class B tetracycline resistance determinant) or C/B (a chimera containing the α domain from class C, the β domain from class B) (see Fig. 1) with an extract containing TetA-6H. A MalE-Tet/TetA-6H mixture was included as a positive control. Since B/B and C/B were not made by cells in sufficient quantities to be detected by Coomassie-stained SDS-PAGE, all proteins bound to TetA-6H were detected by immunoblot of SDS-PAGE gels. The results are shown in Fig. 4. Binding of TetA-6H to Ni-NTA was verified using antiTet (Fig. 4, lane 1'), as was binding of MalE-Tet to TetA-6H (data not shown). Using antiCt, binding of B/B to TetA-6H was seen (Fig. 4, lane 2'), as expected from the earlier experiments. However, little binding of C/B was seen (Fig. 4, lane 3'), even though much more C/B than B/B had been applied to the Ni-NTA columns, (Fig. 4, lane 3 versus lane 2). These results suggested that the α domains of classes B and C interacted poorly. They also unexpectedly implied that there was little interaction between the β domain of class B (on C/B) and the α domain of class B (on TetA-6H).

Use of Tetβ-6H to Confirm Lack of α-β and β-β Interactions: Observation of α-α Interactions—The fact that C/B did not bind to TetA-6H suggested that the α and β domains of class B did not interact, despite genetic data to the contrary. It was possible that the C/B protein was for some reason in a nonbinding conformation after extraction. Therefore, we constructed a polyhistidine fusion having only the β domain of class B for binding studies. This fusion was designated Tetβ-6H.

The Tetβ-6H protein was identified on Coomassie-stained SDS-PAGE gels of Ni-NTA-bound protein as a band migrating slightly more slowly than TetA-6H and not present in fusionless host cells (data not shown). Quantiﬁcation of these bands indicated that cells containing pLY22, encoding Tetβ-6H, produced only about 2% as much fusion protein as did cells bearing pLY17 (encoding TetA-6H).

The functionality of Tetβ-6H encoded by pLY22 was assayed in vivo by the ability to complement TetA having a mutated β domain encoded on a compatible plasmid. Two different compatible mutant plasmids were tested in trans with pLY22 (see "Experimental Procedures"). No plasmid offered tetracycline resistance alone (minimal inhibitory concentration of tetracycline <0.2 μg/ml). pLY22 complemented both mutant plasmids to give tetracycline resistance (minimal inhibitory concentration >10 μg/ml). Therefore, the Tetβ-6H domain was functional, at least in the intact cell expressing a complementing Tet protein.

Biochemical studies were then performed. Extracts of cells containing Tetβ-6H or TetA-6H were loaded onto Ni-NTA columns at volumes which contained approximately equal amounts of each fusion protein. A volume of a host extract identical to the volume used for Tetβ-6H was also loaded onto a column as a control. Then an extract containing Tet279-LacZ (or MalE-Tet in one case) was passed over the columns. Binding of Tet279-LacZ to the host extract column was considered as background. The molar ratio of Tet279-LacZ to 6H fusion applied to the column was about 2. The net molar ratio eluting at 1 M imidazole was about 0.038 for TetA-6H but only 0.002 for

**Fig. 3.** Association of MalE-Tet with Ni-NTA-bound TetA-6H or 6H-IICB9c. Dodecylmaltoside extracts of membranes from 6 A530 units of cells containing MalE-Tet were mixed with the same amount of a second dodecylmaltoside extract (see below) and loaded onto Ni-NTA columns. Proteins which bound and eluted at 40 mM or 1 M imidazole are shown by SDS-PAGE (1.4 A530 unit/lane). The second extract contained: lanes 1, no fusion (host extract); lanes 2, 6H-IICB9c; lanes 3, TetA-6H. Molecular mass standards (kDa) are in lane S. The molar ratio of MalE-Tet : TetA-6H was about 0.9 for the material loaded, 0.5 for the 40 mM eluate, and 0.09 for the 1 M eluate. The molar ratio of MalE-Tet : 6H-IICB9c loaded was 2.0.

**Fig. 4.** Association of B/B but not C/B with Ni-NTA-bound TetA-6H. The method of Fig. 3 was employed except that immunoblots were done. An extract representing 9 A530 units of the TetA-6H strain was mixed with an extract representing 24 A530 units of the other strains. Each SDS-PAGE lane contains 0.075 A530 units (loaded onto Ni-NTA) or 1.7 A530 units (TetA-6H, bound to Ni-NTA). Lanes 1–3 are the loaded samples: 1, host with no plasmid; 2, B/B; 3, C/B. Lanes 1–3' are the bound samples, all containing TetA-6H plus: 1', host with no plasmid; 2', B/B; 3', C/B. Blots were probed with antiCt to reveal B/B and C/B or with antiTet to reveal TetA-6H.


**DISCUSSION**

We report here initial biochemical studies on the quaternary structure of the tetracycline-antibiotic binding protein TetA. From genetic data described earlier we had expected that TetA protein was capable of functioning in vivo as a dimer or other multimer. We had also imagined that the interaction would be between the α and β domains. Earlier we had found that a small proportion of either the B/B protein or the C/B chimeric protein could be cross-linked into a immunoreactive band having the molecular weight of a dimer, but that little coimmunoprecipitation of one Tet polypeptide by antibody specific for the other occurred, with or without cross-linking.

In the present work we explored another biochemical methodology to test the multimer hypothesis. Immobilized Ni²⁺ can be used to bind proteins having a polyhistidine region (23). By the use of TetA-polyhistidine fusion proteins, we were able to clearly show specific association between two distinguishable Tet protein molecules from cell membrane extracts. These heteromultimers between two Tet species formed simply upon mixing a dodecylmaltoside extract containing one Tet species with an extract containing the other. Apparently, in the mixtures the original homomultimers have readily dissociated (within minutes) into subunits, followed by rapid association with a heterologous subunit into a multimer which was stable enough to detect. Presumably, the rates of both association and dissociation are high, while the former exceeds the latter to account for multimer stability on Ni-NTA. Binding did not occur between TetA and another polyhistidine fusion of an integral membrane transport protein, Hc-hicb⁹⁻, nor did other cell membrane proteins associate with Tet-6H to any notable extent, as was evident by its purity following Ni-NTA chromatography.

Therefore, we believe the Tet-Tet interactions to be specific. Unexpectedly the crucial interaction in formation of Tet multimers in vitro appeared to be between two (or more) α domains, rather than between an α and a β domain. However, in intact cells, besides the genetic data there are also physical indications of an α-β interaction. The amount of a polypeptide comprising the β half of TetA in whole cells was increased 1.5-fold or more by the presence of the α half polypeptide, suggesting a physical interaction of the two (14). We have observed that the amount of full-length B/C construct was increased notably if the C/B chimera was present in the same cell; a simple explanation for those results could be that the B/C protein formed a multimer with the C/B protein via same-class α-β interactions and stabilized it, although other explanations are possible. The fact that in the present work we did not see α-β interactions after the Tet protein had been extracted may mean that the β domain for the C/B and Tet-6H constructs did not have native binding properties in our extracts or under our assay conditions. However, recent circular dichroism studies on purified full-length Tet-6H, at least, show that both α and β domains in that polypeptide do have approximately the expected α-helical content.

A TetA dimer may be held together both by α-α interactions (seen in the present study for proteins extracted from membranes by dodecylmaltoside) and by α-β interactions (not apparent using extracts, but inferred from genetic and biochemical studies in whole cells). A model in which both α-α and α-β interactions occur within a TetA dimer is shown in Fig. 5. During complementation of B/C with C/B in vivo, the α-α interactions would presumably not occur, but the α-β ones would. Two active sites/wild type dimer, or one/ complementing dimer, would be expected. Our model might explain why Tetα-6H was found in cells at high concentrations similar to those of the full-length fusion Tet-6H, while the amount of Tetβ-6H was 50-fold lower, since the model allows α to bind to α (or to β), and such associations may prevent degradation. Absence of self-association for β, as modeled, would lead to degradation of β when alone in a cell.

The proposed structure of the dimer differs from that proposed for a monomer both because of the additional α-α interactions and the altered topology of the central loop (Fig. 5). A monomer of TetA has both the domains (α and β) required for activity, and we cannot discount the possibility that a complex consisting of only one α and one β domain is capable of functioning. On the other hand, even when these two domains are tethered together in a normal monomer, considerable interaction with other such monomers must be allowed in vivo, since intermolecular complementation can occur. Self-association of monomers into dimers might be favored in the two-dimensional
membrane bilayer even more than the considerable degree seen here in detergent extracts.

Multimerization provides possibilities for scaffolding, interfaces, and allostery. Some other membrane transport proteins of the same superfamily (24) as TetA are known to occur as multimers, including the facilitated glucose transporter GLUT1 (17, 25), the erythrocyte anion exchanger Band 3 (26), and the Na⁺/glucose cotransporter (27). The relationship between these multimerizations and function is uncertain (25, 28), and at least one example exists (the lactose permease, LacY) in which the transporter almost certainly functions as a monomer (29). Our results strengthen the concept that the mechanism of action of TetA involves a multimeric state.

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