Secretion of thyroglobulin (Tg, a large homodimeric glycoprotein) is essential to deliver Tg to its site of iodination for thyroxine biosynthesis. An L2263P missense mutation in Tg has been proposed as the molecular defect causing congenital goitrous hypothyroidism in cog/cog mice due to perturbed Tg homodimerization, resulting in its retention within the endoplasmic reticulum. The mutation falls within a carboxyl-terminal region of Tg with high structural similarity to the entirety of acetylcholinesterase (AChE), a secretory protein that also forms homodimers. We provide new evidence that authentic AChE and the cholinesterase-like domain of Tg share a common tertiary structure. Moreover, we find that a Tg truncation, deleted of the cholinesterase-like region (but not a comparably sized deletion of internal Tg regions), blocks Tg export. Appending to this truncation a cDNA encoding authentic AChE results in translation of a chimeric protein in which AChE is present in a native, enzymatically active (albeit latent) conformation, and this fully rescues Tg secretion. Introduction of the cog mutation inhibits AChE enzyme activity, and established denaturing mutations of AChE block secretion of the Tg. Additional studies show that the native structure of the AChE region functions as a "dimerization domain," facilitating intracellular transport of Tg to the site of thyroid hormonogenesis.

Thyroid hormones are essential for development, oxidative metabolism, and regulated gene expression in many tissues. Biosynthesis of thyroid hormones is critically dependent upon the native three-dimensional structure of the large homodimeric glycoprotein precursor, thyroglobulin (Tg)\(^1\) (1). Tg, the most highly expressed protein in the normal thyroid gland, is predominantly extracellular, secreted by thyrocytes into the lumen of thyroid follicles (2, 3). Once secreted, a number of tyrosine residues of Tg are iodinated to form moniodothyrosine and diiodothyrosine; this is associated with a coupling reaction between two diiodothyrosine residues to form thyroxine, the major form of thyroid hormone produced in the thyroid gland. The Tg protein is essential to this process in two ways. First, Tg contains within its encoded structure information for efficient coupling of iodotyrosyl residues involved in thyroid hormone biosynthesis (4). Second, Tg must achieve a conformational state sufficient to escape quality control in the endoplasmic reticulum (ER) in order to be exported through the secretory pathway to the site of iodination (5).

Analysis of the Tg monomer primary sequence (amounting to ~2,750 amino acids) suggests that it is subdivided into regional structures (6). The most heavily used site of iodination and thyroxine synthesis occurs within the first 130 residues of the Tg protein (7, 8). While there are other hormonogenic sites on Tg, the foregoing information leaves open the question of the biological significance of much of the remaining 2,620 residues (9, 10). The amino-terminal ~60% of Tg contains the numerous cysteine-rich "Tg type 1 repeats" (11, 12); then a smaller region downstream exhibits a distinct series of cysteine-rich repeats that have little homology to other proteins in existing data bases. Finally the most carboxy-terminal region of Tg exhibits 31% identity and 47% similarity to the entire length of acetylcholinesterase (AChE) (13, 14).

Patients with congenital goitrous hypothyroidism and defective Tg, as well as animal models of the human disease, have been recognized for many years. The thyrocytes in such patients typically exhibit a dilated ER lumen (15, 16) with little or no secreted Tg (17). Mutations in a variety of Tg regions can result in goitrous hypothyroidism as a result of deficiency of Tg folding (10).

Under normal circumstances, Tg makes a homodimer within the ER prior to its export through the secretory pathway (18–21). By contrast, in thyrocytes of cog/cog mice, there is activation of the unfolded protein response resulting in induced expression of multiple ER molecular chaperones, secondary to a temperature-sensitive defect in Tg folding and dimerization (20). The Tg from cog/cog mice fails to homodimerize and cannot be efficiently exported from the ER (22), instead undergoing a slow process of ER-associated degradation (23). Consequently, in cog/cog mice, there is greatly diminished iodination of Tg resulting in insufficient thyroid hormone synthesis (24–26) and congenital goitrous hypothyroidism. The cog Tg secretion defect is associated with a single L2263P substitution that falls within the AChE homology domain (22). Interestingly, authentic AChE also undergoes homodimerization (27–30), which has been reported to enhance its efficiency of export in the secretory pathway (31).

Although there are no x-ray crystallographic data of any Tg regions, we believe that there is reason to suspect that Tg and AChE share certain common tertiary structural features (13, 22, 32–34). For example, the six cysteine residues involved in AChE intrachain disulphide bonding are conserved within Tg (32). AChE additionally possesses one unique carbonyl-terminal unpaired Cys residue, which it can use in creation of a covalent interchain cross-link between monomer subunits in the homodimer (35), whereas Tg homodimers are nonco-
valently associated at the time of secretion. Dimerization of both proteins occurs upon biosynthesis in the ER.

In this report, we begin to explore the role of the AChE-like domain in Tg biosynthesis. Our results support the hypothesis that the AChE-like region of Tg functions as a dimerization domain, facilitating efficient intracellular transport of the much larger Tg molecule, via the secretory pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 or 293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Because of background contributed by serum Tg, for Western blotting experiments, cells were cultured post-transfection in AIM-V medium (Invitrogen) in the absence of serum. Cells were grown to 80% confluency in 6-well plates, and all transfections used LipofectAMINE (Invitrogen) with 2 μg of plasmid DNA. All experiments were performed 48 h after transient transfection. For transfections of Tg and Tg chimeric cDNAs, the pcB6 (cytomegalovirus promoter-driven) expression buffer but without protease inhibitors and then treated for 5 min with 1 U of thermolysin, the cells were lysed in the same buffer but without protease inhibitors and then treated for 5 min with 1 μg/ml aprotinin (diisopropyl fluorophosphate was avoided because of background due to the presence of serum AChE enzyme activity in the media). Thus, each well of cells was lysed in 300 μl of 100 mM NaCl, 1% Triton X-100, 10 mM EDTA, 25 mM Tris, pH 7.5, and a mixture of protease inhibitors including 1.2 μl leupeptin, 1.1 μM pepstatin, 6.5 mM EDTA, 2 μM E64, and 1 μg/ml aprotinin (disopropyl fluorophosphate was avoided as it inhibits hAChE enzymatic activity). One-third of each lysate was then used directly for colorimetric acetylthiocholinesterase activity (36).

Enzymatic Activity of hAChE or yBtgAChE—For measurement of recombinant AChE or yBtgAChE catalytic activity, expression of intracellular rather than secreted activity was measured to eliminate background due to the presence of serum AChE enzyme activity in the media. Thus, each well of cells was lysed in 300 μl of 100 mM NaCl, 1% Triton X-100, 10 mM EDTA, 25 mM Tris, pH 7.5, and a mixture of protease inhibitors including 1.2 μl leupeptin, 1.1 μM pepstatin, 6.5 mM EDTA, 2 μM E64, and 1 μg/ml aprotinin (disopropyl fluorophosphate was avoided as it inhibits hAChE enzymatic activity). One-third of each lysate was then used directly for colorimetric acetylcholinesterase activity (36). For limited proteolysis with thermolysin, the cells were lysed in the same buffer but without protease inhibitors and then treated for 5 min with 1 μg/ml thermolysin at 37 °C, and then the antiprotease mixture was added. Each activity experiment was performed at least three times with mean and standard deviations calculated.

Immunoprecipitation and Western Blotting—Cells were metabolically pulse-labeled for 30 min with 35S-amino acids according to standard protocols and chased in complete growth medium containing serum for the times indicated. Regardless of whether intact bTg or yBtgAChE cDNAs were used for transfection, cell lysates were preclarified and then immunoprecipitated with rabbit polyclonal anti-Tg antibodies using protein A-agarose as immunoprecipitant. SDS-4% PAGE was run under reducing or nonreducing conditions. For Western blotting of secreted Tg, one-half of the experimental medium was first precipitated with trichloroacetic acid, washed, and then dissolved in gel sample buffer prior to SDS-PAGE. A peroxidase-conjugated anti-rabbit was used as secondary antibody followed by enhanced chemiluminescence (ECL, Amersham Biosciences).

RESULTS AND DISCUSSION

There is considerable normal sequence variation between Tg of different species and among Tg from individuals of the same species (10). It seemed possible that the Tg mutation L2266P, reported as the molecular defect causing congenital goitrous hypothyroidism by blocking Tg secretion from thyrocytes of cog/cog mice (22), which falls in the AChE-like domain of Tg, might occur uniquely in the context of the murine Tg sequence. We began by introducing an L2266P missense mutation in the context of bovine Tg. Whereas in transiently transfected COS cells the empty (pcB6) vector produced no Tg and the wild-type bTg cDNA resulted in secreted Tg protein, the encoded proline substitution at the homologous site in bTg produced a protein that could not be secreted from cells (Fig. 1). Like that from cog/cog mice (20), the mutant Tg protein was sensitive to endoglycosidase H (not shown), indicating failure of the protein to reach the medial Gols. The data suggest that structural consequences of this mutation are not limited to murine Tg but might have similar denaturing effects within the other members of the AChE-like superfamily. Specifically, the seven-residue coding sequence surrounding the site of the cog mutation is 100% conserved between AChE and Tg (22). Nevertheless, the conserved Leu residue at the site of the cog mutation has never been studied previously in the context of authentic AChE. We therefore introduced L99P into recombinant hAChE to examine the production of native protein as judged by enzymatic activity in transiently transfected 293 cells. With comparable transfection efficiency, cell lysates expressing recombinant hAChE showed a ~9-fold increase in intracellular esterase activity over endogenous background activity recovered from control cells transfected with empty vector (Fig. 2). After subtraction of background intracellular esterase activity, cells transfected with hAChE cDNA bearing the L99P mutation (homologous to the cog site) showed an ~80% reduction in intracellular esterase activity compared with cells transfected with the wild-type AChE cDNA (Fig. 2). Because of difficulties with Western blotting of intracellular hAChE using commercial antibodies, these particular experiments did not examine the protein expression level of the hAChE-cog mutant; therefore, the diminished activity might reflect effects on protein expression/stability or intrinsic activity (addressed further below). The data indicate that the cog mutation significantly impairs production of active hAChE, raising the possibility that the mutation causes similar perturbation in both Tg and AChE structures.

One of the three disulfide bonds of the AChE-like domain of bTg, conserved with AChE (13, 32, 37), is predicted to employ Cys244-Cys257, the latter residue separated by only two intervening residues from the site encoded by the cog mutation. It has been proposed that Pro replacing Leu at this site, which in AChE is located in a neighboring β-turn, might interfere with formation of that particular disulfide bond (22). To examine the structural significance of this disulfide in bTg export, Cys244 and Cys257 were mutated either individually or in tandem, each of which blocked bTg secretion (Fig. 3A). Another predicted disulfide pair (Cys2571-Cys2607) falls near a helix that
form a four-helix bundle involved in homodimerization of AChE (29, 30). Both of these individual Cys mutants also blocked bTg secretion (Fig. 3B). Moreover, two salt bridges employing Asp^{175} as well as Asp^{404} of hAChE are critically involved in structural stability of hAChE protein (37), and we noted that homologous residues at both sites are present in Tg. Indeed, D2340N and D2568N missense mutations of Tg, respectively, both seriously impaired Tg secretion (Fig. 3C). A diminished recovery of the D2568N mutant at 3 h chase is consistent with a foreshortened intracellular half-life; however, a detailed examination of the disposal of the intracellularly entrapped Tg mutant awaits further investigation. The AChE-like domain of authentic Tg lacks both a critical Ser residue in the catalytic triad as well as conserved flanking residues (Table I); thus, Tg is not expected to encode an active cholinesterase. Nevertheless, multiple distinct point mutations known to adversely affect the native structure of AChE similarly impair Tg secretion (Fig. 3), consistent with the idea that the AChE-like domain of Tg shares a common tertiary structural folding pattern to that of authentic AChE. We therefore wished to test the hypothesis that the AChE-like region provides essential structural information for formation of stable Tg homodimers.

We proceeded to make an internal deletion mutant encoding a Tg protein of ∼2,200 amino acids (“Tg1231–1912”) in order to contrast its secretion to that of a comparably sized Tg mutant truncated at the carboxyl terminus to remove the AChE-like region (“TgΔAChE-like”). The analysis by reducing SDS-PAGE and Western blotting yielded two interesting observations. For one, although the molecular mass of TgΔAChE-like is actually predicted to be slightly (∼100 residues) larger than that of the internal deletion mutant, removal of the AChE-like region produced a glycoprotein with a smaller apparent molecular mass (Fig. 4). As protein disulfides were fully reduced in the analysis and 10% of Tg molecular mass normally is comprised of carbohydrate, these observations suggest that loss of the AChE-like region might result in secondary deficiency in Tg post-translational modifications. In addition, the carboxyl-terminal truncation mutant was not detectably secreted (Fig. 4). Thus, we find that deletion of the AChE-like region alone is sufficient to block Tg transport through the secretory pathway, strongly suggesting that presence of a native AChE-like region is important for the folding and maturation of Tg that leads to escape from ER quality control.

As homodimerization may be important for Tg escape from ER quality control (20), we considered the possibility that TgAChE-like might be rescued in the secretory pathway by appending in-frame to this cDNA a sequence encoding an artificial dimerization domain. Although many potential dimerization domains could be considered, we elected to add back the authentic hAChE-coding sequence in place of the missing AChE-like region for acetylcholinesterase activity. It was predicted that cells expressing bTg alone would exhibit no activity above the background observed in cells transfected with empty vector (Fig. 5A); however, little apparent activity was initially noted upon transfection of the cDNA encoding the bTg:hAChE chimera (Fig. 5A).

We considered the possibility that hAChE in the chimera

![Image](60x231 to 305x481)

**FIG. 2.** Introduction of an L99P mutation homologous to that found in Tg of cog cog mice inhibits the enzymatic activity of AChE. 293 cells transiently transfected with an empty vector (293-con) or with expression plasmids containing the constructs indicated were lysed, and the lysates were measured directly for AChE enzyme activity. Co-transfections with a proinsulin cDNA and measurement by radioimmunoassay were used to demonstrate comparable transfection efficiency between samples. After subtracting the background of endogenous esterase activity in control cells, introduction of the cog mutation caused a 77.5% reduction in intracellular AChE activity in 293-hAChE-cog cells compared with cells with the wild-type AChE cDNA (293-hAChE).

![Image](125x598 to 240x738)

**FIG. 3.** Mutations that cause misfolding of AChE and also impair intracellular transport of Tg. A and B, COS-7 cells were transfected with the constructs indicated and then pulse-labeled, chased, and analyzed as in Fig. 1. mTg, mouse Tg. C, COS-7 cells were transfected with the constructs indicated, pulse-labeled with ^35S-amino acids for 30 min, and chased for 3 h before analysis as in Fig. 1. The mutagenized residues in Tg are listed above the comparable residues in hAChE, shown below. Only the 330-kDa region of the gel is shown. C, cell lysates; M, bathing medium; WT, wild type.

### Table I

| Torpedo AChE       | bovine AChE | human AChE | rat Tg | mouse Tg | bovine Tg |
|--------------------|-------------|------------|--------|----------|----------|
| VTLFGES           | AGSASV     | AGSASV     | GGF    | GGF      | GGF      |
| KXDE              | AKDE       | VKDE       | GSF    | GSF      | GSF      |
|                   |            |            | GVI    | GVI      | GVI      |
|                   |            |            | H      | H        | H        |
|                   |            |            | G      | G        | G        |
|                   |            |            | H      | H        | H        |
|                   |            |            | G      | G        | G        |
|                   |            |            | S      | S        | S        |
|                   |            |            | H      | H        | H        |
|                   |            |            | SS     | SS       | SS       |
|                   |            |            | 2368   | 2497     | 2601     |

**Alignment of sequences surrounding the catalytic triad of AChE** (boxed residues) with those surrounding the homologous sites in Tg

Numbering is provided for the critical residues of Torpedo AChE as well as bTg. Note that Tg in all species lacks the critical Ser residue of the catalytic triad of AChE.
Acetylcholinesterase Homology Region of Tg

**Primary Structure**

| M<sub>r</sub> | Express. | Secret. |
|---|---|---|
| 301 | ++++ | ++++
| 227 | ++++ | ++
| 239 | ++++ | —

**Fig. 4.** Loss of the AcChE-like region blocks Tg secretion. Tg bearing an internal deletion (TgΔ1231–1912) or bearing deletion of the AcChE-like region (TgΔAcChE-like) is shown in schematic form at the top of the figure. These two constructs were transiently transfected into COS-7 cells, and both cell lysates (C) and bathing media (M) were analyzed by SDS-4% PAGE under reducing conditions (100 mM dithiothreitol), electrophoresis, blotting, and immunoblotting with anti-Tg. The position of rat Tg secreted from the FRTL5 cell line (F) is shown in the first lane as a molecular weight marker for size comparison with that of the mutants. Express., expressed; Secret., secreted; wt-Tg, wild-type Tg.

**Fig. 5.** Lack of AChE enzymatic activity of bTg and bTglhAChE in comparison with hAChE. A, 293 cells were transiently transfected with the constructs indicated, and the cell lysates were analyzed for AChE enzyme activity without further treatment. B, 293 cells were transfected with authentic hAChE and then lysed and exposed to either limited proteolysis with thermolysin (1 μg/ml for 5 min at 37 degrees C) or heating to 50 degrees C (for 5 min) before measurement of AChE enzymatic activity. Values shown are the mean ± S.D. from at least three independent experiments.

**Fig. 6.** AChE is folded to a native (active) but latent conformation within the Tg structure. A, 293 cells were transfected with the constructs indicated and exposed to limited proteolysis with thermolysin as in Fig. 5B before measurement of AChE enzymatic activity. B, 293 cells were transfected with the constructs indicated and exposed to 50 degrees C (for 5 min) as in Fig. 5B before measurement of AChE enzymatic activity. Note that both thermolysin treatment and 50 degrees C heat treatment denature upstream Tg domains sufficiently to liberate latent catalytic activity from the bTghAChE chimera. Values shown are the mean ± S.D. from at least three independent experiments.

It was important to know whether the structure of Tg allows sufficient exposure of the AChE contact zone to allow dimerization in this region. As noted above, both AChE and Tg homodimerize, but authentic AChE uniquely contains a short carboxyl-terminal extension with one extra unpaired cysteine that allows some homodimers to form an intersubunit disulfide bond (35), providing a convenient dimerization assay (30). Therefore, the secretion from metabolically labeled 293 cells expressing bTg or bTglhAChE was immunoprecipitated with anti-Tg and analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 7A). As expected, the mobility of monomeric 330-kDa bTg or bTglhAChE is faster under nonreducing conditions because of the presence of the many intrachain disulfide bonds (39). More importantly, under nonreducing conditions, a disulfide-linked bTghAChE dimer band appeared (Fig. 7A, arrow). Furthermore, when compared with Tg lacking its own AChE-like region (Fig. 4), the addition of authentic AChE clearly restored Tg secretion (Fig. 7B). Important to our earlier considerations, introduction of the cog mutation within the bTghAChE chimera did not diminish protein expression but did cause the protein to be retained intracellularly, recreating the biochemical phenotype for Tg in cog/cog thyrocytes. Taken together, the data indicate that normal conformational maturation of the AChE-like region facilitates homodimerization and intracellular transport of Tg to the site of its iodination for thyroid hormonogenesis.

Because hAChE is folded in a catalytically active conformation, this domain provides a unique folding reporter at the carboxyl-terminal end of Tg. Thus, replacement of the AChE-like region with authentic hAChE creates interesting possibili-
ties for future studies of upstream Tg regions. Specifically, there are additional mutations residing in these regions that may cause Tg export defects leading to congenital goitrous hypothyroidism in humans (17). The presence of latent AChE enzyme activity at the carboxyl terminus of Tg should permit distinction of those upstream mutations that result in only local domain-specific or regional misfolding versus those that propagate misfolding down the polypeptide chain into the AChE region. Moreover, homodimerization of these mutants can be readily screened for future studies of upstream Tg regions. Specifically, there were helpful discussions during the course of this work.

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FIG. 7. Fusion of authentic hAChE at the carboxyl terminus of Tg lacking its own AChE-like region restores Tg homodimerization and secretion, which is blocked by introduction of the cog mutation. A. 293 cells were either untransfected (Con) or transfected to express wild-type Tg or bTgAChE. The cells were pulse-labeled for 30 min and chased for 3.5 h, and the chase media were immunoprecipitated with anti-Tg and analyzed by SDS-PAGE under reducing or nonreducing conditions as indicated. Each lane is run in duplicate with approximately a 2-fold difference in intensity. In the nonreduced gel, the bTgAChE monomer band is decreased in intensity concomitant with the appearance of covalently linked homodimers (marked with an arrow). B. 293 cells were transfected with either empty vector or bTgAChE cDNA without or with the cog mutation, as indicated, and pulse-labeled, chased, and then analyzed as in A. Note that a non-native hAChE region cannot support secretion of Tg.
