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A putative role for tissue transglutaminase in Alzheimer's disease pathology.

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A PUTATIVE ROLE FOR TISSUE TRANSGLUTAMINASE IN ALZHEIMER'S DISEASE PATHOLOGY

by

MICHAEL LESLIE MILLER

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
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ABSTRACT OF DISSERTATION
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Title A Putative Role for Tissue Transglutaminase in Alzheimer's Disease Pathology

Alzheimer disease is a progressive neurodegenerative disease which involves a loss of cognition, memory, language, judgement, and behavior. The neuronal degeneration seen in Alzheimer disease is accompanied by the formation of intraneuronal inclusions known as neurofibrillary tangles, which consist of large aggregates of paired helical filaments (PHFs). The primary proteinaceous constituent of these PHFs is an abnormally phosphorylated form of the microtubule associated protein τ. Although τ is typically soluble, the PHFs contain τ in an insoluble, aggregated state that is resistant to proteolysis. The biochemical properties of PHFs suggest that a covalent cross-linking of τ may occur in Alzheimer disease. Tissue transglutaminase is a calcium-dependent cross-linking enzyme found in a variety of cell types, including neurons. As there are several reports of calcium dysregulation in Alzheimer disease, it is reasonable to hypothesize that increases in intracellular calcium concentrations may pathologically activate tissue transglutaminase, leading to a cross-linking of τ into PHFs. The current studies are designed to assess the tissue transglutaminase-mediated cross-linking of τ as a possible mechanism of neurofibrillary tangle formation in Alzheimer disease. The first study demonstrates that τ is an excellent substrate of tissue transglutaminase in vitro, displaying kinetic parameters that are potentially physiological. Bovine τ and recombinant human τ isoforms rapidly form high molecular weight, cross-linked polymers upon incubation with tissue transglutaminase, and cross-linked τ has an appearance similar to τ from Alzheimer disease brains when visualized on a western blot. The next study investigates protein levels and activity of tissue transglutaminase in Alzheimer disease and control postmortem brain tissue. In Alzheimer disease, both the levels and the activity of tissue transglutaminase are sig-

nificantly elevated over controls in the prefrontal cortex, which is affected in Alzheimer disease, but not in the cerebellum, which is generally spared. The third study provides a protocol for isolating tissue transglutaminase-mediated ε-(γ-glutamyl)lysine isopeptide bonds from cross-linked substrates, using exhaustive proteolytic digestion, derivatization, and separation with high performance liquid chromatography. Taken together, these studies support the hypothesis that tissue transglutaminase may be involved in the formation of insoluble τ aggregates in Alzheimer disease, and suggest experiments that may be used to investigate the potential involvement of tissue transglutaminase in other paradigms.

DEDICATION

Of science and nature and forces unseen,
Concepts evoke unpretentious delight;
Elicit desire, stimulate curiosity
Quench the hunger, while feeding the flame
A driving passion which summons us all
To meet the Maker and see his hands at work:
Thus has he created us to be,
Each of us, in our own way, the purest scientist

The passions run deep, far beyond this domain
To fields and forests, lakes and streams
To look upon these, in each can be seen
Incomplete visions of the Creator
Tiny splinters comprising a whole
When taken together;
To neglect a piece is to miss a slice of creation,
A slight to the artist
Who lovingly crafted each tiny shard

A wandering heart and a restless soul
Together find richness in every pursuit:
A reverent mind seeking expression
Through prayer, proscenium, pencil or song
In each craft, praise through a different voice
Attempting to match the Voice from within

Regret not that life can be lived only once
For to those who are truly alive
One life is enough.

-Michael L. Miller
1/10/96

This dissertation is dedicated to my parents, who have always encouraged my creativity, challenged me to succeed, and faithfully supported me in every endeavor.

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INTRODUCTION

Alzheimer disease is a progressive neurodegenerative disease that involves a gradual loss of cognitive functions, leading to deficits in memory, language, judgement, and behavior (McKhann et al., 1984). In the United States alone, it is responsible for more than 100,000 deaths per year, thereby making it the fourth leading cause of death among the elderly (Hardy and Allsop, 1991). It affects an estimated 4 million people in the U.S., including up to 50% of those over the age of 85 (Evans et al., 1989; Evans, 1990; Hebert et al., 1995).

The neuronal degeneration seen in late stages of the disease is accompanied by the formation of pathological structures known as neurofibrillary tangles, found in certain susceptible populations of neurons in the hippocampus, neocortex, and amygdala (Goedert et al., 1991; Braak and Braak, 1996). At the ultrastructural level, neurofibrillary tangles consist of a vast array of paired helical filaments (PHFs), which appear to be two 10-nm filaments wrapped around each other in a left-handed helix, with a half-periodicity of 80 nm, a width that alternates between 8 and 20 nm, and a length of 500-2000 nm (Crowther and Wischik, 1985; Wischik et al., 1985; Wischik and Crowther, 1986; Crowther, 1991). Isolated PHFs have unique biochemical properties, such as resistance to proteolysis (Kondo et al., 1988; Wischik et al., 1988*a*; Jakes et al., 1991) and insolubility in harsh solvents, denaturants, and reducing agents (Selkoe et al., 1982*b*). Since these properties of PHFs make them difficult to analyze, their full characterization is incomplete. However, it appears that they are composed primarily, if not entirely, of an abnormally phosphorylated form of the τ protein, known as PHF-τ (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986; Goedert et al., 1988; Kondo et al., 1988; Flament et al., 1989; Greenberg and Davies, 1990; Lee et al., 1991).

τ protein

τ is a family of closely related microtubule-associated phosphoproteins which is localized predominantly (although not specifically) in the axo-somatic compartment of neurons (Binder et al., 1985; Papasozomenos and Binder, 1987). Under normal physiological conditions, τ exists as a soluble, cytoplasmic protein and is involved in the nucleation, polymerization, and stabilization of microtubules (Weingarten et al., 1975; Cleveland et al., 1977*a*,*b*; Drubin and Kirschner, 1986; Knops et al., 1991; Brandt and Lee, 1993*a*). When τ is injected or transfected into cells that are normally devoid of this protein, it localizes specifically to the microtubule network, causes increases in cytosolic microtubule density, and dramatically slows the depolymerization of microtubules when the cells are subsequently treated with nocodazole (Drubin and Kirschner, 1986; Lee and Rook, 1992). τ also appears to be involved in the initiation and/or stabilization of neuronal morphology (Kosik and Caceres, 1991; Kwei et al., 1998), as the expression of τ correlates with neurite outgrowth in PC-12 and cultured neuronal cells (Drubin et al., 1985; Ferreira et al., 1989), and inhibition of τ expression leads to suppressed neurite elongation in primary cerebellar neurons (Caceres and Kosik, 1990). Furthermore, the overexpression of τ in nonneuronal insect cells induces the formation of elaborate processes (Knops et al., 1991). τ protein levels form a concentration gradient along the axons of developing neurons, with maximal levels being located in the distal tips near the growth cones (Black et al., 1996; Kempf et al., 1996). The phosphorylation of τ follows an inverse distribution, as phosphate-dependent antibodies detect high levels of phosphorylated τ in the soma, but mostly dephosphorylated τ in the growth cone (Mandell and Banker, 1996). The presence of these gradients, coupled with the fact that τ is not found in dendritic processes, suggests that τ may be involved in the establishment of neuronal polarity (Mandell and Banker, 1996).

Human τ consists of multiple isoforms, ranging in molecular mass from 37-46 kDa, which are alternatively spliced variants of a single gene, located on chromosome 17 (Goedert et al., 1989*a*,*b*; Himmler, 1989; Murrell et al., 1997). As shown in Fig. 1, the τ

isoforms contain either three (T3) or four (T4) tandem repeats of 31-32 amino acids in the carboxy-terminal half of the protein, generated by the exclusion or inclusion of exon 10 (Goedert et al., 1989*b*; Goedert and Jakes, 1990). These repeats are homologous and are often referred to as microtubule-binding domains because initial evidence indicated that these were the regions that enabled τ to bind to microtubules (Aizawa et al., 1988; Ennulat et al., 1989; Himmler et al., 1989; Lee et al., 1989*a*; Butner and Kirschner, 1991) Recent evidence suggests that this may be a misnomer, however, and that, although the repeat regions are important for the polymerization and stabilization of microtubules, it may actually be the regions in between, or on either side of the repeats, that are involved in the binding per se (Goode and Feinstein, 1994; Gustke et al., 1994; Mandelkow et al., 1995*b*; Goode et al., 1997).

Mature human τ isoforms may also contain additional inserts of 29 (designated as "S") or 58 (designated as "L") amino acid segments near the amino terminus (Goedert et al., 1989*a*; Goedert and Jakes, 1990). These isoforms are generated by the inclusion of exon 2 or of exons 2 and 3, respectively. Thus, six isoforms have been identified in human brain, with fetal isoform T3 being the shortest and isoform T4L being the longest. The function of the amino-terminal domain of τ is unclear; however, it has been shown to mediate the interaction of τ with the plasma membrane (Brandt et al., 1995) and may also serve as a spacer between microtubules (Chen et al., 1992). No functional significance has yet been determined for the amino terminal inserts; however, the different isoforms have complex and differential effects on microtubule binding, polymerization, and bundling (Scott et al., 1991; Lee and Rook, 1992; Scott et al., 1992; Brandt and Lee, 1993*b*; Gustke et al., 1994). The expression of each isoform appears to be dependent upon the cell type, the stage of development, and the localization of τ within the cell (Goedert and Jakes, 1990; Collet et al., 1997; Uberti et al., 1997).

The primary structure of τ contains many basic amino acids, and the τ isoforms have pI values ranging between 8.65 (T4L) and 10.05 (T3). The sequence of τ includes

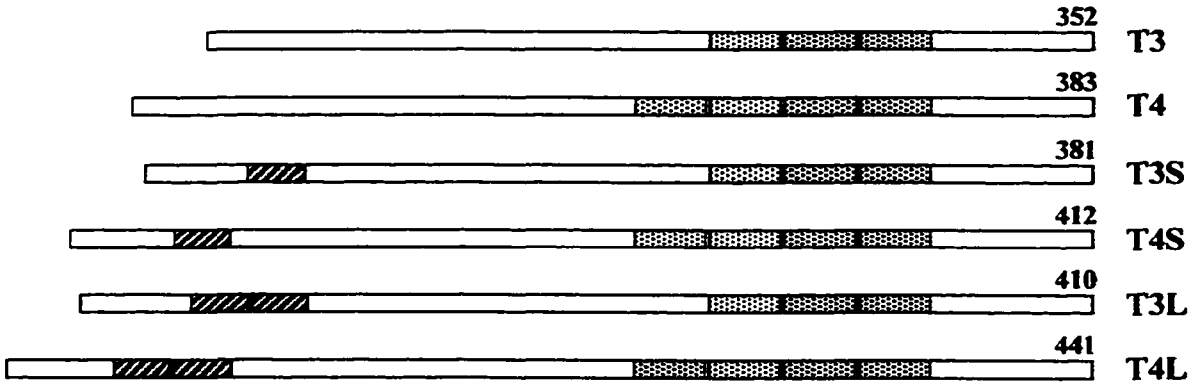

FIG. 1. Human tau isoforms. Schematic representation of the six human tau isoforms. Microtubule-binding repeat regions are represented by shaded boxes, and amino-terminal inserts are shown as stippled boxes. Isoform names and the number of amino acids in each isoform are indicated on the left side of the diagram.

several glutamine (19 in T4L, and 14 in T3) and lysine (44 in T4L, and 37 in T3) residues, a consideration which is relevant to this proposal. τ has very little defined secondary structure and is primarily a thin, elongated molecule as determined by primary structure predictions, circular dichroism, and metal shadowing techniques (Cleveland et al., 1977*a*; Lee et al., 1988; Wille et al., 1992). X-ray scattering studies indicate that even in solution it has no well-defined size or shape, which is indicative of a random ("Gaussian") polymer (Schweers et al., 1994; Mandelkow et al., 1995*a*). Thus, τ behaves as if it were primarily denatured, even in buffered solutions (Schweers et al., 1994; Mandelkow et al., 1995*a*). Probably as a result of its conformation, τ is heat stable and shows retarded electrophoretic mobility relative to the mobility predicted by its deduced amino acid sequence (Cleveland et al., 1977*a*,*b*; Lee et al., 1988; Himmler et al., 1989). Although τ has little secondary structure, it readily self-associates in vitro to form dimeric and polymeric aggregates, including highly-ordered filamentous structures (Hagestedt et al., 1989; Jakes et al., 1991; Crowther et al., 1992; Wille et al., 1992; de Ancos et al., 1993; Crowther et al., 1994). The τ monomers dimerize in an antiparallel orientation, and the interaction involves the repeat regions of the molecule (Jakes et al., 1991; Crowther et al., 1992; Wille et al., 1992; de Ancos et al., 1993; Crowther et al., 1994).

Phosphorylation is a very important posttranslational modification of τ. τ can be phosphorylated in vitro by almost every protein kinase studied thus far, presumably because of its loose structure and the presence of many serine and threonine residues (Mandelkow et al., 1995*b*). Adult rat τ has been shown to be predominantly phosphorylated at five sites in vivo, while fetal rat τ has been shown to be phosphorylated at up to 12 sites (Watanabe et al., 1993; Morishima-Kawashima et al., 1995*a*). The extensive phosphorylation of τ during neuronal development (Brion et al., 1993; Goedert et al., 1993; Morishima-Kawashima et al., 1995*b*; Burack and Halpain, 1996) may be involved in regulating cytoskeletal rearrangements during mitosis (Jicha et al., 1997*b*; Illenberger et al., 1998). Adult human τ also appears to be extensively phosphorylated in vivo, albeit to a

low stoichiometry, and most of these phosphorylation sites become rapidly dephosphorylated after biopsy (Matsuo et al., 1994; Jicha et al., 1997*b*). Hyperphosphorylation of τ is implicated in Alzheimer disease, where as many as 25 phosphorylation sites have been identified (Hasegawa et al., 1992; Morishima-Kawashima et al., 1995*b*; Hanger et al., 1998). The phosphorylation of τ has several putative effects, including decreasing its ability to bind, nucleate, polymerize, and/or stabilize microtubules (Lindwall and Cole, 1984; Biernat et al., 1993; Bramblett et al., 1993; Alonso et al., 1994; Utton et al., 1997; Illenberger et al., 1998); decreasing its electrophoretic mobility in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Grundke-Iqbal et al., 1986; Bramblett et al., 1993); decreasing its pI (Greenberg et al., 1992); inducing a conformational change in which τ is longer and stiffer (Hagestedt et al., 1989); inhibiting its susceptibility to proteolysis (Litersky and Johnson, 1992, 1995; Vincent et al., 1994); increasing its susceptibility to further phosphorylation (Singh et al., 1995; Sengupta et al., 1997; Zheng-Fischhofer et al., 1998); changing its localization (Papasozomenos and Binder, 1987); and either increasing (Alonso et al., 1994) or decreasing (Guttmann et al., 1995) its ability to self-associate. Conversely, dephosphorylation of phosphorylated τ restores its normal microtubule-binding properties and electrophoretic mobility (Lee et al., 1991; Greenberg et al., 1992; Bramblett et al., 1993). τ may be phosphorylated on tyrosine residues as well, a modification which could relate to its high concentration in growth cones during neurite outgrowth (Lee et al., 1998).

In addition to phosphorylation, τ is likely to be modified by other co- or post-translational events. Several studies have indicated that glycosylation and glycation (the enzymatic and nonenzymatic covalent attachment of carbohydrates to proteins, respectively), may play a role as well. Bovine τ has been shown to be modified at several serine and threonine residues in vivo by O-linked N-acetylglycosamination, a posttranslational process which may compete for normal phosphorylation sites (Arnold et al., 1996). Although no cotranslational N-linked glycosylation has been detected in normal τ, it has been shown that

PHF tangles and soluble τ from Alzheimer disease brains contain N-linked sugars that may contribute to the helical nature of the PHFs (Wang et al., 1996). In addition, several lysine residues have been shown to be in vitro sites of nonenzymatic glycation by reducing sugars (Nacharaju et al., 1997), and glycated τ has been detected in PHFs of Alzheimer disease (Ledesma et al., 1994). The PHFs may also contain covalently-attached glycolipids consisting of glucose polymers associated with esterified fatty acids (Goux et al., 1996). The presence of ubiquitin in PHFs also suggests that τ may be ubiquitinated under certain conditions (Morishima-Kawashima et al., 1993).

τ in Alzheimer Disease

In brain tissue from patients with Alzheimer disease, τ is found in many different states. On one end of the spectrum is a subset of τ which is unaffected by the disease and is the same as normal, soluble τ from control brains. On the other end of the spectrum is the insoluble τ found in neurofibrillary tangles, which has its own distinct biochemical properties (discussed below). Other populations of τ appear to create a continuum between the normal, soluble state and the tangle state. The differences between these types of τ appear to be dependent upon posttranslational modifications, particularly phosphorylation. Several different forms of τ which can be isolated in Alzheimer disease are discussed below.

τ is the predominant protein component of the neurofibrillary tangles found in Alzheimer disease brain. Unfortunately, the nomenclature used to distinguish various populations of τ in Alzheimer disease is somewhat ambiguous, so in this work τ from neurofibrillary tangles will be referred to as NFT-τ, even though this designation is not in common usage. As indicated above, NFT-τ is in the form of large, insoluble aggregates of intertwined PHFs, which can accumulate in mass until they consume the entire cell body of a neuron. Neurofibrillary tangles are insoluble in detergents, acids, bases, and chaotropic agents (Selkoe et al., 1982*b*) and are also resistant to proteolysis (Kondo et al.,

1988; Wischik et al., 1988*a*; Jakes et al., 1991), and to date there is no known procedure for resolving these aggregations into their constituent τ monomers. Biochemical and immunological studies of neurofibrillary tangles indicate that NFT-τ is highly phosphorylated (see below) and may contain many of the other co- and posttranslational modifications mentioned above. It has also been reported that one or more lysine residues may be modified in τ isolated from PHFs (Wischik et al., 1988*b*; Liu et al., 1991; Nieto et al., 1991), a property which is relevant to this work. Neurofibrillary tangles represent τ in its most insoluble, highly modified state.

Aside from the neurofibrillary tangles, it is also possible to isolate τ from Alzheimer disease brains in the form of SDS-soluble PHFs. These soluble PHFs are structurally similar to the insoluble PHFs, except that they can be dispersed into monomeric τ with 2% SDS or 8 M guanidine hydrochloride (Greenberg and Davies, 1990). It has been hypothesized that soluble PHFs are uncondensed precursors of the insoluble PHFs found in neurofibrillary tangles (Goedert et al., 1992; Greenberg et al., 1992; Iqbal et al., 1994), although the mechanisms underlying such a conversion to the insoluble state are unknown. τ which is isolated from soluble PHFs is commonly referred to as PHF-τ (Greenberg and Davies, 1990), although unfortunately no distinction is usually made between it and the condensed form of τ found in neurofibrillary tangles. In this manuscript, τ isolated from soluble PHFs will be designated PHF-τ, with the understanding that some further step or steps may be necessary to convert it into the insoluble form of NFT-τ.

Compared to τ from normal controls, PHF-τ from Alzheimer disease brain is extensively phosphorylated. As many as 25 phosphorylation sites have been identified in PHF-τ, including 11 on Ser/Thr-Pro motifs (Hanger et al., 1998). This hyperphosphorylation causes a significant decrease in the mobility of PHF-τ on SDS-polyacrylamide gels (Grundke-Iqbal et al., 1986; Greenberg and Davies, 1990; Lee et al., 1991; Greenberg et al., 1992) and also changes the immunoreactivity of τ to certain antibodies. Both of these properties enable PHF-τ to be differentiated from normal τ and are often used

for diagnostic purposes (Greenberg and Davies, 1990; Lee et al., 1991; Greenberg et al., 1992; Matsuo et al., 1994; Braak and Braak, 1995). Yet, although these properties are useful diagnostic tools, the actual role of phosphorylation in the etiology of the disease is unclear. It has been suggested that phosphorylation is an underlying event in PHF formation (Grundke-Iqbal et al., 1986; Flament et al., 1989; Lee et al., 1991; Kopke et al., 1993). However, this hypothesis has recently been challenged (Bondareff et al., 1995; Lai et al., 1995; Wischik et al., 1995), and it is difficult to ascertain whether phosphorylation is a necessary or sufficient step in the self-association of τ and its conversion to the insoluble state. Dephosphorylation of soluble τ from Alzheimer disease brain restores its normal microtubule-binding properties and electrophoretic mobility (Lee et al., 1991; Greenberg et al., 1992; Bramblett et al., 1993).

A significant event in Alzheimer disease pathology appears to be the self-association of τ monomers into straight filaments and PHFs, a phenomenon which is probably concomitant with the depolymerization of microtubules. Several groups have attempted to resolve the conditions required for soluble τ monomers to form filamentous structures in vitro. Initial studies with τ paracrystals indicated that τ can form filamentous structures in a head-to-tail orientation, but the filaments from these experiments were linear rather than helical (Lichtenberg et al., 1988; Hagestedt et al., 1989). Next, it was discovered that PHF-like structures can be assembled from the truncated repeat regions of τ (Crowther et al., 1992; Wille et al., 1992) or from full-length τ (Crowther et al., 1994), but such studies utilized crystallization techniques to force τ into self-associating. Attempts to create PHF-like filaments under near-physiological conditions were largely unsuccessful until it was discovered that heparin and other sulfated glycosaminoglycans stimulate the formation of straight and PHF-like τ filaments in solution (Goedert et al., 1996; Perez et al., 1996). Other polyanionic substances, such as RNA (Kampers et al., 1996; Hasegawa et al., 1997), DNA (Hasegawa et al., 1997), and the polyglutamate domain of tubulin (Friedhoff et al., 1998), were shown to have similar effects, and many of these polyanionic

substances also induce the phosphorylation of τ and/or inhibit its binding to microtubules (Goedert et al., 1996; Kampers et al., 1996). The treatment of actual PHFs from Alzheimer disease brain with either heparinase (Arrasate et al., 1997) or an endo-glycosidase (Wang et al., 1996) causes them to lose their helical nature and form straight filaments, implying that sulfated glycosaminoglycans and/or glycosylation are involved in stabilizing the structure of the PHF. Other groups have also been successful at creating straight filaments of τ using free fatty acids (Wilson and Binder, 1997) or τ that has been glycated (Ledesma et al., 1996).

It is the interest of this lab to investigate the mechanisms whereby normal τ becomes converted to the state of τ found in neurofibrillary tangles. It is clear that τ readily self-associates and that PHF-like filaments of τ can be formed under certain conditions, but these soluble PHFs are neither SDS-insoluble nor resistant to proteases. Thus far, attempts to mimic the formation of neurofibrillary tangle-like structures have been unsuccessful, and it appears that there is an additional step in the reaction mechanism which has previously been overlooked. One possibility is that a covalent cross-linking enzyme interacts with the τ dimers and polymers, stabilizing the interaction and seeding further polymer formation (Selkoe et al., 1982*a*,*b*; Miller and Anderton, 1986).

Tissue transglutaminase

The transglutaminases are a family of calcium-activated, thiol dependent enzymes which can cross-link substrate proteins into high molecular weight complexes that are resistant to proteolysis and insoluble in detergents, denaturants, and reducing agents (Folk and Finlayson, 1977). Five distinct transglutaminase genes have been identified in mammals, including blood coagulation factor XIIIa, epidermal, keratinocyte, prostate, and tissue type transglutaminase (Fesus et al., 1996). Various transglutaminases are involved in skin, hair, and nail cornification; hepatic fibrogenesis; fibrin cross-linking during clot

formation; and the formation of cataracts in the eye lens (for a review, see Greenberg et al., 1991; Fesus et al., 1996; Melino and Piacentini, 1998).

Tissue (cellular, or type II) transglutaminase (EC 2.3.2.13) is an inducible enzyme which is widely distributed in a variety of cells (see Fesus and Thomazy, 1988; Fesus et al., 1996). Typically considered a soluble, cytoplasmic protein, it can also be detected in particulate fractions (Gilad and Varon, 1985; Slife et al., 1985; Chang and Chung, 1986; Hand et al., 1993), in the nucleus (Lesort et al., 1998), and in the extracellular matrix (Aeschlimann et al., 1992, 1995). Tissue transglutaminase has been implicated in a diverse range of processes, such as cell adhesion and extracellular matrix formation (Slife et al., 1986; Aeschlimann et al., 1992; Gentile et al., 1992; Perry et al., 1995; Borge et al., 1996; Ueki et al., 1996; Jones et al., 1997), receptor mediated endocytosis (Davies et al., 1980), activation of midkine (Kojima et al., 1997), cataract formation (Lorand et al., 1981; Clement et al., 1998), enhancement of phospholipase A2 activity (Cordella-Miele et al., 1990, 1993), and nerve regeneration after injury (Gilad et al., 1985; Chakraborty et al., 1987; Tetzlaff et al., 1988; Eitan and Schwartz, 1993). In some cell types, tissue transglutaminase is also involved in apoptosis or programmed cell death (for a review, see Fesus et al., 1996; Melino and Piacentini, 1998). As a cell death protein, its induction late in apoptosis likely leads to the cross-linking of membrane proteins into a rigid shell which maintains cell integrity, enabling phagocytosis to occur without an inflammatory immune response (Fesus et al., 1987, 1989; Knight et al., 1991; Piacentini et al., 1991; Perry and Haynes, 1993). Recent evidence indicates that tissue transglutaminase may also be induced early in the apoptotic pathway, where its activation is thought to be a central factor in the apoptotic mechanism (Melino et al., 1994; Melino and Piacentini, 1998).

There is substantial evidence to indicate that tissue transglutaminase is involved in neuronal processes. Tissue transglutaminase has been immunocytochemically localized within neurons (Miller and Anderton, 1986; Perry et al., 1995) and is abundant in axons and synaptosome fractions (Gilad and Varon, 1985; Pastuszko et al., 1986; Facchiano et

al., 1993; Perry et al., 1995). Significant tissue transglutaminase activity has been demonstrated in the frontal and temporal neocortex, hippocampus, cerebellum, and white matter of human brain (Selkoe et al., 1982*a*; Gilad and Varon, 1985). Increases in tissue transglutaminase activity can be seen in developing brain, where it may be involved in neurite outgrowth (Gilad and Varon, 1985; Maccioni and Seeds, 1986; Perry et al., 1995; Kojima et al., 1997) or neuronal differentiation through certain pathways (Hand et al., 1993). Neuronal functions of tissue transglutaminase may also include the formation of focal adhesions (Perry et al., 1995; Chowdhury et al., 1997), cytoskeletal stabilization (Maccioni and Seeds, 1986; Chowdhury et al., 1997), and modulation of neurotransmitter release (Pastuszko et al., 1986; Facchiano et al., 1993). Tissue transglutaminase activity can be upregulated in neurons by depolarizing agents (Ando et al., 1993; Ando and Nagata, 1993) and by neuronal injury (Gilad et al., 1985; Tetzlaff et al., 1988), and tissue transglutaminase is believed to be responsible for the isodipeptide bonds induced in hippocampal slices by long-term potentiation (Friedrich et al., 1991). Endogenous labeling of proteins by tissue transglutaminase-dependent mechanisms has been demonstrated in situ in the giant cholinergic neuron of *Aplysia* (Ambron and Kremzner, 1982). Several neuronal substrates of tissue transglutaminase have been identified, including tubulin and microtubules, actin and neurofilaments, myelin basic protein, microtubule-associated proteins, synapsin I, and β/A4 amyloid (Selkoe et al., 1982*b*; Maccioni and Seeds, 1986; Miller and Anderton, 1986; Facchiano et al., 1993; Ikura et al., 1993; Dudek and Johnson, 1994; Rasmussen et al., 1994; Nemes et al., 1997).

The human tissue transglutaminase gene can be spliced into at least three different gene products, containing 695, 548, or 349 amino acids (Fraij et al., 1992; Fraij and Gonzales, 1996, 1997). As shown in Fig. 2, the full-length human tissue transglutaminase protein contains a fibronectin binding site, a nucleotide binding domain, an active-site cysteine residue, one or more putative calcium-binding motifs, and a phospholipase C interaction domain (Im et al., 1997; Aeschlimann et al., 1998). The smaller isoforms of

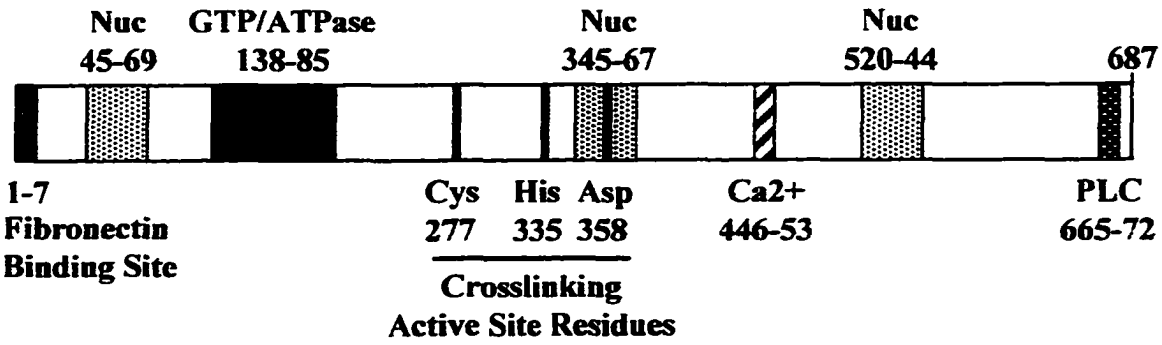

FIG. 2. Proposed functional domains of tissue transglutaminase. The active site cysteine residue also requires the charged amino acids histidine and asparagine, which form a catalytic triad (Aeschlimann et al., 1998). GTPase and ATPase activity appear to be restricted to a segment between amino acids 138-185 (Iismaa et al., 1997), although other nucleotide-binding domains (Nuc) have been identified which may inhibit tissue transglutaminase activity (Takeuchi et al., 1992). Other domains are adapted from Im et al. (1997).

tissue transglutaminase, which can be induced by treatment with retinoic acid, contain differences in the carboxy-terminal ends of the proteins (Fraij and Gonzales, 1997). Analysis of the 5' untranslated region of the human tissue transglutaminase gene indicates the presence of several promoters and potential transcription factor-binding sites, including CAAT and TATA sequences, four SP1 binding sites, four NF1 half-sites, an AP-1 binding site, a potential AP-2 site, an interleukin-6 (IL-6) response element, and a glucocorticoid response element (Lu et al., 1995; Fraij and Gonzales, 1997). In addition, the tissue transglutaminase gene contains a nuclear factor-κB (NFκB) binding site (Mirza et al., 1997) and a novel retinoid response element, consisting of three hexanucleotide "half-sites" that work in concert to bind certain retinoid receptor dimers (Nagy et al., 1996; Yan et al., 1996). The TATA and SP1 binding sites appear to responsible for the constitutive expression of tissue transglutaminase in a wide variety of cells, and this constitutive expression appears to be enhanced by the presence of the CAAT sequence (Lu et al., 1995) and by demethylation of the promoter (Lu and Davies, 1997). The other regulatory sites enable tissue transglutaminase to be induced by a wide variety of factors, including interleukin-6 (Suto et al., 1993), prostaglandin E2 (Ishitani et al., 1988; Mastino et al., 1992), arachidonic acid (Ishitani et al., 1988), transforming growth factor β (George et al., 1990), tumor necrosis factor α (TNF-α) (Kuncio et al., 1998), NFκB (Mirza et al., 1997), sodium butyrate (Birckbichler et al., 1985; Byrd and Lichti, 1987), dexamethasone (Johnson et al., 1998), and retinoic acid (Piacentini et al., 1988*a*; Melino and Piacentini, 1998). Retinoic acid induction is potentiated by cyclic AMP (Murtaugh et al., 1986; Maddox and Haddox, 1988) and appears to induce expression of the smaller isoforms of tissue transglutaminase in certain systems (Fraij et al., 1992; Fraij and Gonzales, 1996).

Interestingly, the name "transglutaminase" is somewhat of a misnomer, as it is not free glutamines but rather peptide-bound γ-glutaminyl residues occupying endo-positions which serve as substrates for the enzyme (Lorand and Conrad, 1984). The mechanism of transglutaminase activity is a two-step, modified double displacement reaction. The

binding of calcium by transglutaminase exposes an active-site cysteine residue which can react with the γ-carboxamide of a substrate glutamine, forming an intermediate γ-glutamyl thioester and releasing ammonia (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Greenberg et al., 1991). The acyl-enzyme intermediate then reacts with a nucleophilic primary amine, resulting in an incorporation of the amine-containing "donor" into the substrate glutamyl "acceptor." If the primary amine is donated by the ε-amino group of a peptide bound lysine, the result is a cross-linking of the two proteins via the formation of a ε-(γ-glutamyl)lysine isopeptide bond (see *Transglutaminase Cross-linking of the Tau Protein*, Fig. 1, reaction A). Alternately, the amine can be donated by histamine or a polyamine (such as spermine, spermidine, cadaverine, or putrescine), resulting in polyamine incorporation and the formation of a (γ-glutamyl)polyamine bond (see *Transglutaminase Cross-linking of the Tau Protein*, Fig. 1, reaction B). It is also possible for a polyamine to act as an N,N-bis(γ-glutamyl)polyamine cross-linking bridge between two glutamine-containing acceptor proteins (Folk et al., 1980; Piacentini et al., 1988*b*). In the absence of a primary amine, transglutaminase reacts with water, resulting in the deamidation of glutamine to glutamate (Folk and Finlayson, 1977; Lorand and Conrad, 1984).

Energetically, the reactions of transglutaminase occur with very little change in free energy: a γ-amide bond is broken (on the glutamine residue), and a γ-amide bond is formed (with the primary amine group) (Lorand and Conrad, 1984). Thus, the reactions of transglutaminase should be considered reversible. Under physiological conditions, however, the immediate protonation of the amino leaving-group helps drive the reaction forward, and resulting phenomena such as clotting and phase transitions make the reversibility of cross-linking reactions difficult to assess (Lorand and Conrad, 1984). A decreased accessibility of transglutaminase to the cross-linked product may render the transglutaminase cross-linking reaction essentially irreversible in vivo, although recent studies with fluorescent transglutaminase substrates suggest that some reversibility may be

possible (Parameswaran et al., 1997). Furthermore, polyamine exchanges by the enzyme are likely to occur (Lorand and Conrad, 1984; Parameswaran et al., 1997).

The specificity of transglutaminase is dependent upon both the structure of the glutamine-containing acceptor (Doyle et al., 1990; Parameswaran et al., 1990; Coussons et al., 1992; Facchiano and Luini, 1992) and the nature of the amine donor (Lorand et al., 1968; Gross et al., 1977; Lorand and Conrad, 1984; Lorand et al., 1992; Grootjans et al., 1995). The reaction mechanism of transglutaminase is a highly specific one, owing to the fact that three macromolecules (two substrates and the enzyme) must come together in a highly oriented fashion during catalysis (Folk and Finlayson, 1977). It can be postulated that, in order to be cross-linked by transglutaminase, substrates must first be oriented in a specific conformation, wherein the lysine residue of the donor protein is closely apposed to the substrate glutamine residue of the acceptor protein (Greenberg et al., 1991). Such an orientation is needed to ensure that the lysine is incorporated rather than another primary amine. If this is the case, then only substrate proteins which are already "self-associated" in a specific arrangement have the potential to be cross-linked by the enzyme.

A key step in the reaction mechanism is the binding of calcium ions to transglutaminase and exposure of the active site cysteine (Lorand and Conrad, 1984; Greenberg et al., 1991). The apparent *Km* for the activation of tissue transglutaminase by calcium in vitro is approximately 3-4 μM (Hand et al., 1985; Hand et al., 1988), although required calcium concentrations in vivo may be much higher due to the presence of several inhibiting factors found inside the cell. Although this may appear to be outside the physiological range, the presence of the lipid sphingosylphosphocholine has been shown to reduce the calcium requirement severalfold, and other tissue transglutaminase-enhancing factors may exist as well (Lai et al., 1997, 1998). It has been shown that muscarinic cholinergic agonists which release calcium from the endoplasmic reticulum can cause the activation of tissue transglutaminase, indicating that activation can occur in response to physiological stimuli and at physiological calcium concentrations (Zhang et al., 1998). Also, as the

determination of the apparent *Km* depends upon the nature of the substrate utilized--nearly always N,N'-dimethylcasein (Hand et al., 1985; Lai et al., 1997)--it is possible that the actual *Km* is significantly lower. A recently reported "superactivation" of tissue transglutaminase may actually be due to substrate modifications which enhance the affinity of tissue transglutaminase-substrate binding, rather than affecting the tissue transglutaminase itself (Groenen et al., 1993; Holmes and Haynes, 1996). The affinity of tissue transglutaminase for its substrates can potentially be either enhanced or decreased by prior posttranslational modifications of the substrate, such as phosphorylation, methylation, or modification by free radicals (Lorand and Conrad, 1984; Groenen et al., 1993).

The activity of tissue transglutaminase can be inhibited by zinc (but not magnesium) ions, which interfere with calcium binding, and by disulfide-containing metabolites (e.g., cystamine) which interfere with the active site of the enzyme (Lorand and Conrad, 1984; Hand et al., 1985). Cross-linking activity can be inhibited by high concentrations of polyamines, which compete for the acyl-enzyme intermediate (Lorand and Conrad, 1984). Nucleotides also play complex roles in the regulation of tissue transglutaminase activity. Although the tissue transglutaminase sequence does not contain a typical nucleotide-binding consensus sequence, it has been demonstrated that tissue transglutaminase can bind and cleave both ATP and GTP, and that micromolar concentrations of GTP cause a reversible, noncompetitive inhibition of enzyme activity (Achyuthan and Greenberg, 1987; Lee et al., 1989*b*; Takeuchi et al., 1992; Fraij, 1996; Lai et al., 1998). Recent studies have shown that the binding sites for ATP and GTP are separate and that the binding of GTP causes a conformational change which inhibits tissue transglutaminase activity (but not ATPase activity) and protects tissue transglutaminase from proteolysis (Achyuthan and Greenberg, 1987; Lai et al., 1998; Zhang et al., 1998). The binding of ATP, which occurs with a three-fold greater affinity than the binding of GTP, does not inhibit tissue transglutaminase activity, but does inhibit GTP hydrolysis (Lai et al., 1998). Furthermore, the inhibition of tissue transglutaminase activity by GTP can be partially reversed by increased calcium ion

concentration (Achyuthan and Greenberg, 1987; Parameswaran et al., 1997; Lai et al., 1998). The GTP and ATP hydrolysis sites appear to be localized between amino acids 138-185 (Iismaa et al., 1997), although other putative nucleotide binding sites have been identified which may play a role in the inhibition of tissue transglutaminase activity (see Fig. 2) (Takeuchi et al., 1992).

Interestingly, another function of tissue transglutaminase has recently been reported which helps explain the sensitivity of tissue transglutaminase to nucleotides: that of a signal-transducing GTP-binding protein. Although it is well-established that the hetero-trimeric GTP-binding proteins $G_{q/11}$ couple to the α1-adrenergic receptor, there is also data to suggest that tissue transglutaminase can interact with certain α1-receptor subtypes (B and D) and act as a signal-transducing GTP-binding protein (Im et al., 1997). When Nakaoka et al. (1994) sequenced Gαh (the alpha subunit of a G-protein which mediates the signal between the α1-adrenergic receptor and phospholipase C-δ1), they found it to be identical to tissue transglutaminase. Further studies revealed that Gαh/tissue transglutaminase is an atypical, pertussis toxin-insensitive G-protein, which does not contain common GTP-binding motifs (see Im et al., 1997). Yet it binds and cleaves GTP, couples with a variety of receptors, and stimulates the activation of PLC-δ1 (Nakaoka et al., 1994; Hwang et al., 1995; Feng et al., 1996; Im et al., 1997; Lai et al., 1998). The Gαh/tissue trans-glutaminase protein is not considered to be a standard heterotrimeric G-protein because no β- or γ-subunits have been found (Im et al., 1997); however, it appears to be associated with a 50 kDa regulatory protein which may mediate its binding of GTP (Baek et al., 1996).

Thus, the physiological function of tissue transglutaminase appears to be extremely complex. Under certain circumstances, the enzyme may be involved in normal cell signalling (as a G-protein) where it is partially responsible for the viability of the cell. Under other circumstances, or in different cellular compartments, tissue transglutaminase may lose its G-protein function and behave as a cross-linking enzyme (e.g., during

apoptosis). These two functions of Gαh/tissue transglutaminase are probably distinct, as the active sites for each activity are separate, and mutating the active site cystine of tissue transglutaminase has no effect upon its G-protein function (Lee et al., 1993; Chen et al., 1996; Im et al., 1997). It is also possible that Gαh/tissue transglutaminase can rapidly convert from one function to the other, depending upon the conditions of the cell. For example, in certain developing cells, the addition of phenylephrine (an α1 agonist) causes a suppression of apoptosis, correlates with the binding of GTP by Gαh/tissue transglutaminase, and is suggestive of the G-protein function of the enzyme (Gill et al., 1998). If phenylephrine is removed, GTP-binding to Gαh is decreased, and the loss of signal transduction correlates with both the onset of tissue transglutaminase activity and apoptosis (Gill et al., 1998). Under such regulation, "life" and "death" would not be separate events, but rather a continuum, regulated in part by both activities of Gαh/tissue transglutaminase. It has been speculated that, due to the relatively high *K*m of calcium binding and the inhibition by normal levels of nucleotides, tissue transglutaminase may not be active as a cross-linking enzyme in most viable cells (Melino et al., 1994; Smethurst and Griffin, 1996; Lai et al., 1997). However, a wide range of physiological states probably exists within a cell for both functions of Gαh/tissue transglutaminase. Different stages of development and the presence of extracellular and intracellular signals may evoke different properties of this multifunctional enzyme, by stimulating or suppressing its expression, changing its localization, or manipulating the microenvironments that surround it (Lai et al., 1998; Melino and Piacentini, 1998).

Transglutaminase in Alzheimer disease

As tissue transglutaminase is a calcium-activated enzyme (Folk and Finlayson, 1977), a disruption of calcium homeostasis is likely to result in dysregulation of the enzyme. There is substantial evidence to suggest that concentrations of cytosolic calcium are abnormally high in Alzheimer disease. Fibroblasts from Alzheimer disease patients

have been found to contain increased concentrations of bound, cytosolic, and free calcium (Peterson and Goldman, 1986). Increased concentrations of free calcium may also result from decreased levels of calcium-binding and calcium-buffering proteins, as decreased levels of calbindin are found in regions of Alzheimer disease brain which are vulnerable to the disease (Sutherland et al., 1993). Conversely, neurons which contain the calcium-buffering protein parvalbumin appear to be resistant to degeneration in Alzheimer disease (Hof et al., 1991). The high levels of calcium seen in Alzheimer disease would likely stimulate the cross-linking function of tissue transglutaminase, and could possibly disrupt its role as a G-protein, impairing normal cell signalling and causing further damage.

Studies by Mattson and colleagues have suggested that sustained or increased levels of intracellular calcium can also result in cytoskeletal changes, including the depolymerization of microtubules, an accumulation of τ in neuronal somata, and neuronal degeneration (Mattson et al., 1991; Mattson, 1992, 1995). It has been proposed that the accumulation of nonmicrotubule-bound τ in the soma exposes it to kinases which cannot normally access τ, and unusually high τ concentrations may lead its self-aggregation (Mattson, 1992; Mattson, 1995). Influxes of calcium in cultured neurons can also lead to increased immunoreactivity with the Alz-50 antibody, a monoclonal antibody which specifically stains Alzheimer disease brain sections but not controls (Wolozin et al., 1986), giving further support to the hypothesis that increased levels of calcium are involved in Alzheimer disease pathology (Mattson, 1990). Interestingly, τ proteins which have been cross-linked by tissue transglutaminase in vitro also show increased immunoreactivity with Alz-50 (Dudek and Johnson, 1993). Thus, increased levels of intracellular calcium may potentially be involved in the depolymerization of microtubules, the aggregation and hyperphosphorylation of τ, and the activation of tissue transglutaminase.

The pathology of Alzheimer disease may also involve oxidative stress and free radical production (see Blass et al., 1990; Mattson, 1995), a feed-forward processes of cellular damage which leads to increased levels of intracellular calcium through a variety of

mechanisms (Ames et al., 1993; Beal et al., 1993; Coyle and Puttfarcken, 1993; Olanow, 1993; Reiter, 1995). Cultured cortical neurons subjected to oxidative stress undergo apoptosis (Ratan et al., 1994), a tissue transglutaminase-related process which may contribute to neuronal death in Alzheimer disease (Heintz, 1993; Johnson, 1994). Oxidative stress and reactive oxygen intermediates have been shown to activate NFκB (Baeuerle, 1991), a nuclear binding factor which can strongly upregulate tissue transglutaminase mRNA levels (Mirza et al., 1997). High intracellular calcium concentrations could also result in a tissue transglutaminase-mediated enhancement of phospholipase A_2 activity (Cordella-Miele et al., 1993), thereby stimulating further free radical production through the release of arachidonic acid and other polyunsaturated fatty acids (Chan and Fishman, 1980; Coyle and Puttfarcken, 1993). Thus, tissue transglutaminase has the potential to be involved in more than one step of the degenerative process. Interestingly, oxidative stress may also be involved in generating τ pathology in Alzheimer disease, as lipid peroxidation in rat hippocampal neurons generates a byproduct (4-hydroxynonenal) which binds directly to τ and prevents its dephosphorylation (Mattson et al., 1997).

Another prevalent aspect of Alzheimer disease is inflammation and reactive gliosis (Lue et al., 1996; Rogers et al., 1996; McGeer and McGeer, 1997). Recent studies indicate that induction of the inflammatory response has a higher correlation with synapse loss and dementia in Alzheimer disease than other pathological markers and that control subjects with extensive Alzheimer disease lesions but no markers of inflammation displayed no signs of dementia (Lue et al., 1996). At least 20 epidemiologic studies and a small clinical trial indicate that anti-inflammatory drugs can inhibit the onset and progression of Alzheimer disease (McGeer and McGeer, 1997). As tissue transglutaminase can be induced by a number of inflammatory cytokines, including TNF-α and IL-6 which are dramatically upregulated in Alzheimer disease brain (Rogers et al., 1996; McGeer and McGeer, 1997), it is reasonable to expect that tissue transglutaminase levels would increase under such conditions (Suto et al., 1993). Furthermore, activated microglia produce

oxygen free radicals and various neurotoxic agents, which could lead to increases in calcium concentrations and the potential activation of tissue transglutaminase. Interestingly, studies with aging mice indicate that macrophages from senescent mice express higher basal levels of tissue transglutaminase than macrophages from young mice. Furthermore, after the removal of an inflammatory insult, the incremental decrease in tissue transglutaminase activity is lower in old mice than in young (Lavie and Weinreb, 1996).

Other data supports the hypothesis that tissue transglutaminase is involved in Alzheimer disease as well. It has been reported that there are altered levels of tissue transglutaminase activity in Alzheimer disease (Kawashima et al., 1989), as well as alterations in polyamine levels (Morrison and Kish, 1995). The major byproduct of tissue transglutaminase activity is the ammonium ion, and several studies suggest an abnormal accumulation of ammonia in the central nervous system of Alzheimer disease patients (see Seiler, 1993). Amino acid analysis reveals that NFT-τ contains one or more modified lysine residues, a phenomenon which could be explained by the presence of tissue transglutaminase-mediated cross-links (Wischik et al., 1988*b*; Hasegawa et al., 1992). Degenerating neurons from Alzheimer disease hippocampus exhibit increased immunoreactivity to tissue transglutaminase antibodies, and tissue transglutaminase staining co-localizes to the same neurons which display immunoreactivity to the AD-specific antibody PHF-1 (Appelt et al., 1996). Antibodies to tissue transglutaminase also decorate PHFs isolated from Alzheimer disease brains (Appelt and Balin, 1997), consistent with the fact that tissue transglutaminase often incorporates itself into high molecular weight aggregates (Birckbichler et al., 1977; Appelt and Balin, 1997; Chowdhury et al., 1997). Furthermore, a recent study of 10 clinical therapeutic agents currently used in Alzheimer disease indicates that all but one of them had an inhibitory effect on the cross-linking of β-amyloid by tissue transglutaminase (Zhang et al., 1997).

Because tissue transglutaminase catalyzes the formation of ε-(γ-glutamyl)lysine isopeptide bonds between substrate proteins, the resulting cross-linked protein complexes

are insoluble (Folk and Finlayson, 1977), like the neurofibrillary tangles. Indeed, the idea that tissue transglutaminase may be involved in the formation of neurofibrillary tangles is not a novel one. However, the early tissue transglutaminase studies, carried out prior to the knowledge that neurofibrillary tangles were composed primarily of τ, focused on neurofilaments as the potential substrate and produced equivocal results (Selkoe et al., 1982*a*; Miller and Anderton, 1986).

Because of the unique properties of tissue transglutaminase activation and catalysis, we hypothesize that tissue transglutaminase is involved in converting τ from its normal, soluble form to a polymerized, insoluble form, such as that found in the neurofibrillary tangle. It is hypothesized that, in Alzheimer disease, soluble PHFs which contain τ in an ordered, antiparallel state (Ksiezak-Reding and Yen, 1991; Wille et al., 1992) are pathologically exposed to increased calcium levels and abnormal tissue transglutaminase activity, resulting in the ordered, insoluble aggregations of τ found in neurofibrillary tangles.

TRANSGLUTAMINASE CROSS-LINKING OF THE τ PROTEIN

by

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ABSTRACT

Tissue transglutaminase (EC 2.3.2.13) is a calcium-activated enzyme that cross-links specific substrate proteins into insoluble, protease-resistant, high molecular weight complexes. Because the neurofibrillary tangles in Alzheimer disease have similar biochemical characteristics, and because the microtubule-associated protein τ is the predominant component of these structures, the substrate properties of τ with respect to transglutaminase were investigated. Bovine τ and recombinant human τ isoforms rapidly form high molecular weight, cross-linked polymers upon incubation with transglutaminase. Polyamine incorporation assays indicate that bovine τ is an excellent substrate of transglutaminase, with a *K*m of 10.4 ± 2.2 μM and a Vmax of 40.9 ± 4.5 nmol/mg enzyme/min. Individual recombinant human τ isoforms are not equivalent with respect to transglutaminase, as the smallest isoform T3 (352 amino acids) is not as good a substrate as the larger isoforms T4 (383 amino acids) and T4L (441 amino acids). To determine which segments of the τ protein are susceptible to modification by transglutaminase, τ was labeled with [^{3}H]-putrescine by transglutaminase and proteolyzed with α-chymotrypsin, and the breakdown products were analyzed. These experiments demonstrate that the enzyme modifies τ at only one or a few discrete sites, primarily in the carboxyl half of the molecule. Thus the reaction is specific for only a small number of the many glutamine residues in τ. Furthermore, a τ deletion construct (T264) containing a portion of the microtubule binding domains, which is a substrate of transglutaminase, cannot be cross-linked by the enzyme. This provides evidence that the cross-linking reaction is specific, and requires that the substrates be appropriately associated for cross-linking to occur.

INTRODUCTION

One of the primary hallmarks of Alzheimer disease is the accumulation of neurofibrillary tangles in the soma of affected neurons in the neocortex and hippocampus (Goedert et al., 1991*a*; Goedert, 1993). Neurofibrillary tangles are composed of paired

helical filaments (PHFs), which are resistant to proteolysis (Kondo et al., 1988; Wischik et al., 1988; Jakes et al., 1991), and insoluble in harsh solvents, denaturants, and reducing agents (Selkoe et al., 1982*b*). This characteristic insolubility of the PHFs in neurofibrillary tangles has been of interest to researchers for some time. More than 10 years ago, it was proposed that these insoluble PHFs arise from abnormal covalent cross-linking of constituent proteins at nondisulfide-bonded amino acid residues (Selkoe et al., 1982*a*; Miller and Anderton, 1986). Although it was originally believed that PHFs were composed of neurofilaments (Gambetti et al., 1981; Sternberger et al., 1985), it has subsequently been determined that PHFs are composed primarily, if not entirely, of an abnormally phosphorylated form of the τ protein, known as PHF-τ (Kosik et al., 1986; Wood et al., 1986; Goedert et al., 1988; Greenberg and Davies, 1990; Lee et al., 1991).

τ is a family of closely related microtubule-associated phosphoproteins found primarily in the axons and cell bodies of neurons (Papasozomenos and Binder, 1987). The six τ isoforms found in adult human brain are generated by alternative mRNA splicing of a single gene and contain either three or four microtubule-binding domains in the carboxy-terminal half of the molecule (Goedert et al., 1989). Additionally, mature isoforms can contain inserts of 29 or 58 amino acids near the amino terminus (Goedert et al., 1989). Normally, τ is a soluble protein that is involved in the polymerization and stabilization of microtubules (Drubin and Kirschner, 1986; Knops et al., 1991). However, in Alzheimer disease, τ becomes localized to the somato-dendritic compartment as well as the axon, is abnormally phosphorylated, loses its ability to polymerize microtubules efficiently (Lu and Wood, 1993), and assembles into PHFs and insoluble neurofibrillary tangles (for a review, see Goedert, 1993). The mechanisms and interrelationships of these events are unknown. Protease digestion studies have indicated that neurofibrillary tangles consist of an insoluble PHF core that contains the carboxyl 40% of the τ protein and a "fuzzy outer coating" around the core, formed by the amino terminus of τ projecting away from the core (Wischik et al., 1988; Ksiezak-Reding and Yen, 1991). It is also possible to isolate

sodium dodecyl sulfate (SDS)-soluble PHFs from Alzheimer disease brains, and these PHFs are also composed of PHF-τ (Greenberg and Davies, 1990; Lee et al., 1991). These soluble PHFs may be uncondensed precursors of the insoluble PHFs found in neurofibrillary tangles (Crowther et al., 1992; Goedert et al., 1992); however, the mechanisms underlying such a conversion to the insoluble state are unknown.

One enzyme that has the potential to be involved in the aggregation of τ and its conversion into insoluble neurofibrillary tangles is tissue transglutaminase (EC 2.3.2.13). The transglutaminases are a family of calcium-activated enzymes that cross-link substrate proteins into high molecular weight complexes that are resistant to proteolysis and insoluble in detergents, denaturants, and reducing agents (Folk and Finlayson, 1977). Specific transglutaminases are involved in the cornification of skin, hair, and nails, the cross-linking of fibrin in clot formation, and the formation of cataracts in the eye lens (for a review, see (Greenberg et al., 1991).

Tissue transglutaminase is primarily a cytosolic enzyme found in various organ-specific cells. It has been immunocytochemically localized within neurons (Miller and Anderton, 1986), and is abundant within nerve terminals (Gilad and Varon, 1985; Facchiano et al., 1993). Significant transglutaminase activity has been demonstrated in the frontal and temporal neocortex, hippocampus, cerebellum, and white matter of human brain (Selkoe et al., 1982*a*). Tissue transglutaminase is an inducible enzyme which is up-regulated both during neuronal development (Gilad and Varon, 1985; Maccioni and Seeds, 1986) and in apoptosis, or programmed cell death (Fesus et al., 1987, 1989; El Alaoui et al., 1992), and it has recently been shown to modify synapsin I, another neuron-specific phosphoprotein (Facchiano et al., 1993). As transglutaminase catalyzes the formation of ε-(γ-glutamyl)lysine isopeptide bonds between substrate proteins rather than disulfide bonds, the resulting cross-linked protein complexes are insoluble (Folk and Finlayson, 1977), like the neurofibrillary tangles. Indeed, the idea that transglutaminase may be involved in the formation of neurofibrillary tangles is not a novel one. However, the early

transglutaminase studies, performed before it was known that tangles were composed primarily of τ, focused on neurofilaments as the potential substrate and produced equivocal results (Selkoe et al., 1982*a*; Miller and Anderton, 1986).

Considering these previous findings, the extent to which τ is a substrate for transglutaminase was investigated. A preliminary study from this laboratory suggested that τ is able to be cross-linked by transglutaminase (Dudek and Johnson, 1993); however, substrate properties of τ were not determined. In this study it is demonstrated that τ is an excellent substrate for transglutaminase, based on both kinetic experiments and biochemical analysis. Although all of the τ isoforms investigated are cross-linked by transglutaminase, kinetic studies demonstrate that they are not equivalent substrates of the enzyme. Furthermore, evidence is presented that transglutaminase modifies τ at only a small number of specific glutamine residues, as determined by proteolytic experiments with recombinant human τ isoforms. The primary site or sites modified by transglutaminase occur in the carboxy half of the τ molecule.

EXPERIMENTAL PROCEDURES

Materials

Guinea pig liver tissue transglutaminase, casein N,N-dimethylated (DMC), bovine serum albumin (BSA), putrescine dihydrochloride, and α-chymotrypsin were purchased from Sigma (St. Louis, MO, U.S.A.); phenylmethylsulfonyl fluoride (PMSF) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.); and [1,4(n)-^{3}H] putrescine dihydrochloride (1 mCi/mL) was purchased from Amersham (Arlington Heights, IL, U.S.A.). EN^{3}HANCE autoradiography enhancer for tritium was purchased from DuPont (Boston, MA, U.S.A.), UniverSol scintillation fluid was purchased from ICN (Costa Mesa, CA, U.S.A.), and the Enhanced Colloidal Gold Total Protein Detection Kit was purchased from Bio-Rad (Hercules, CA, U.S.A.). τ from bovine brains was purified as previously described (Johnson et al., 1989). Recombinant human τ isoforms

and a deletion construct containing amino acids 264-386 (T264; amino acid numbering based on the largest human t isoform: Goedert et al., 1989) were generously provided by C. Scott of Zeneca, Inc. (Scott et al., 1991, 1992). The isoforms used in this study contained either three (T3), or four (T4) microtubule-binding domains in the carboxy-terminal half of the molecule, or four microtubule-binding domains and a 58 amino acid insert near the amino terminus (T4L) (Goedert et al., 1989). Autopsy samples of the prefrontal cortex of 10 Alzheimer disease patients and nine age-matched controls (male and female, ages 57-88) were obtained from the UAB Brain Resource Program.

Antibodies

The following τ antibodies were used in this study: 5E2, T46.1, T14, and τ-1 (Kosik et al., 1988), Alz-50 (Goedert et al., 1991*b*), τ-2 (Watanabe et al., 1992), and 7C11 and 8C11 (Vigo-Pelfrey et al., 1995). Other antibodies included the trans-glutaminase antibody CUB 7402 (Birckbichler et al., 1985), and a monoclonal antibody to ubiquitin, purchased from Zymed. The titers used are indicated in the text.

Immunoblotting of samples from human brain

Samples of autopsy tissue from the prefrontal cortex of Alzheimer disease patients and age-matched controls were homogenized in Laemmli sample buffer without dye, sonicated, and incubated in a boiling water bath for 5 min. Protein concentrations were determined using the method of Lowry et al. (1951), after acid precipitation of the protein. Samples were then further diluted into Laemmli buffer with or without 8 M urea, and equivalent amounts of protein were run on an 8% SDS-polyacrylamide gel, transferred onto nitrocellulose (Towbin et al., 1979), and immunoblotted with antibodies to the τ protein or to ubiquitin.

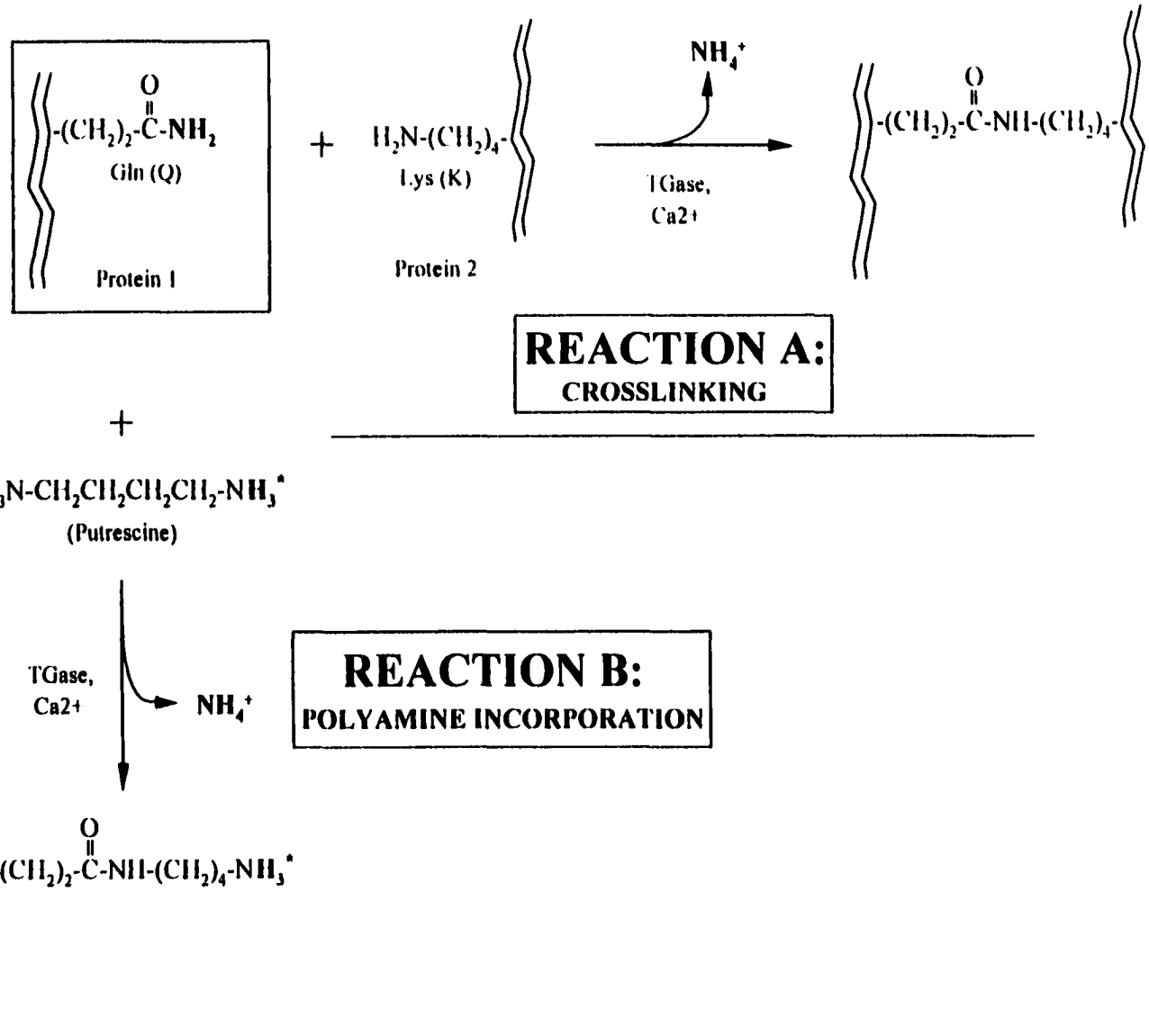
τ cross-linking

Purified bovine τ, human τ isoforms T3, T4, and T4L, and the construct T264 (2.5 μM) were cross-linked individually by tissue transglutaminase (0.25 μM) in a buffer containing 50 mM Tris (pH 8.6) and 5 mM $CaCl_2$ (see Fig. 1, reaction A). Samples were incubated at 37°C for the times indicated, and the reactions were stopped by the addition of Laemmli sample buffer. Samples were placed in a boiling water bath for 5 min, run on an 8% SDS-polyacrylamide gel, transferred onto nitrocellulose (Towbin et al., 1979), and immunoblotted with antibodies to τ, as indicated. T264 was visualized by colloidal gold staining, as specified by the manufacturer's instructions. Micromolar concentrations of the τ isoforms were determined from their predicted molecular masses, and an average molecular mass of 42,000 daltons was used for bovine τ (Himmler, 1989).

Putrescine incorporation assay

The specificity of the transglutaminase reaction was determined by measuring the incorporation of [^{3}H]-putrescine into potential substrates (see Fig. 1, reaction B). Reactions were performed in triplicate, in a buffer containing 50 mM Tris (pH 8.6), 1 mM $CaCl_2$, 20 mM dithiothreitol, and 12.5% glycerol (modified from Achyuthan and Greenberg, 1987). Initial experiments contained 250 μM unlabeled putrescine (Achyuthan and Greenberg, 1987) experiments contained 250 μM unlabeled putrescine (Achyuthan and Greenberg, 1987); however, it was discovered that a high degree of τ self-association and cross-linking was inhibiting putrescine incorporation, and the concentration of unlabeled putrescine was subsequently increased to 1 mM (see below). This higher concentration of putrescine was used in all assays, for both τ and DMC. Transglutaminase (0.25 μM, final concentration) and [^{3}H]-putrescine (2 μCi) were added to each tube and the samples were incubated at 37°C for 10 min. The reactions were stopped by adding an equal volume of 50% trichloroacetic acid (TCA), and proteins were precipitated on ice for 15 min. Samples

FIG. 1. Calcium-dependent reactions of transglutaminase. Schematic diagram of two competing transglutaminase reactions. Transglutaminases are calcium-dependent enzymes which react with free glutamine (Q) residues on substrate proteins (box in upper left). On forming a complex with the glutamine residue and releasing ammonia, the enzyme-substrate intermediate reacts with an appropriate, nearby primary amine. This can be either the ε-amino group of lysine on a nearby protein, resulting in cross-linking of the two proteins (reaction A), or the primary amino group of a polyamine, such as putrescine, resulting in polyamine incorporation into the substrate (reaction B).



were collected on Whatman GF/B filters and rinsed with 5% cold TCA. Filters were dried and the incorporation of [^{3}H]-putrescine into the proteins was determined by liquid scintillation spectrometry. Putrescine incorporation, initial velocities, and stoichiometries of incorporation were calculated for each protein concentration, and data were analyzed using the Michaelis-Menten equation. Results obtained for bovine τ were compared to the results of the same assay performed on DMC, one of the best transglutaminase substrates known to date (Facchiano et al., 1993). All data were analyzed using the Student's *t* test, and values were considered significantly different when $p < 0.05$.

Proteolysis of radiolabeled τ

To determine which segments of the τ molecule are susceptible to modification by transglutaminase, τ was labeled with [^{3}H]-putrescine by transglutaminase, proteolyzed, and the breakdown products were analyzed. Human τ isoforms T3 and T4 (0.1 μg/μl) were incubated at 37°C with transglutaminase (0.25 μM) and [^{3}H]-putrescine (250 μCi/mL, final concentration), in the same buffer used for the putrescine incorporation assay, above. After 30 min, α-chymotrypsin (1 μg/ml, final concentration), which preferentially cleaves proteins on the carboxyl side of aromatic amino acids (Tyr, Phe, and Trp) was added to the reaction and proteolysis was allowed to proceed for an additional 30 min. As a control, transglutaminase alone (0.25 μM) was incubated with α-chymotrypsin under the same conditions. Reactions were stopped by the addition of PMSF (2 mM, final concentration) and Laemmli sample buffer. Samples were incubated in a boiling water bath for 5 min, and run on a 12.5% SDS-polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue, impregnated with EN^{3}HANCE autoradiography enhancer, dried, and exposed to x-ray film. Aliquots of the same samples were also transferred onto nitrocellulose as above and probed with antibodies to τ or transglutaminase. The same experiments were also performed with unlabeled putrescine, and the resulting blots were immunolabeled or double labeled with one or more antibodies to τ, as indicated.

RESULTS

High molecular weight aggregates of τ in Alzheimer disease frontal cortex

Immunoblots of human prefrontal cortex homogenates, from Alzheimer disease patients and age-matched controls, were probed with antibodies to the τ protein. τ immunoreactivity indicated that the prefrontal cortex of Alzheimer disease patients contains a form of τ that migrates as a high molecular weight "smear" of immunoreactivity above the bands of monomeric τ (Fig. 2). Although the smearing is generally seen only in Alzheimer disease brain (Fig. 2), it is occasionally found, to a much lesser extent, in certain samples from age matched controls (data not shown). Such a smearing of τ immunoreactivity in Alzheimer disease was reported previously (Morishima-Kawashima et al., 1993), and a subset of this τ was reportedly conjugated with ubiquitin. When the immunoblots from control and Alzheimer disease brain were probed with a monoclonal antibody to ubiquitin, high molecular weight ubiquitin-reactive bands and "smears" were also observed, but the extent to which the ubiquitin immunoreactivity overlapped the τ immunoreactivity was minimal (data not shown). However, the high molecular weight smearing of τ in Alzheimer disease had a similar appearance to bovine τ, which had been incubated in vitro with transglutaminase (Fig. 2, and also see Dudek and Johnson, 1993). Although this result does not necessarily implicate transglutaminase in Alzheimer disease, the intriguing similarity led us to investigate the extent to which τ is a substrate for transglutaminase.

Putrescine incorporation assay

As shown in Fig. 1, transglutaminase catalyzes two competing nucleophilic displacement reactions: protein-protein cross-linking and the incorporation of polyamines into protein substrates. In the presence of excess polyamines, transglutaminase will catalyze the incorporation of a polyamine (reaction B), rather than a protein-bound lysine (reaction A) into a substrate protein.

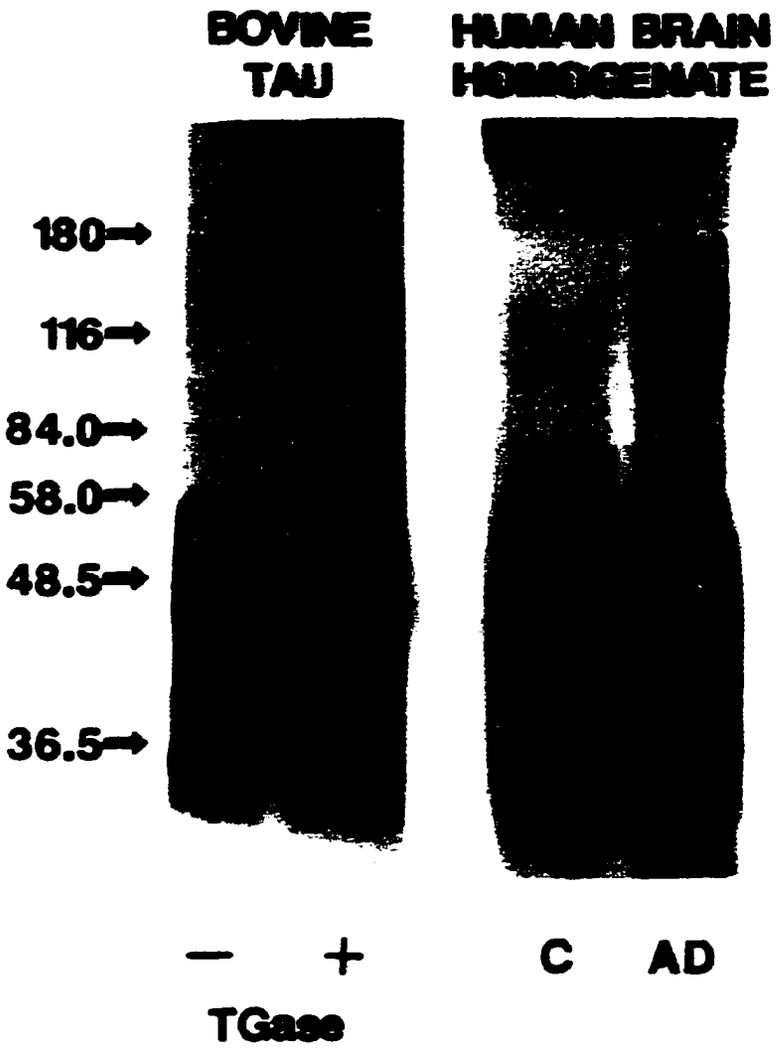


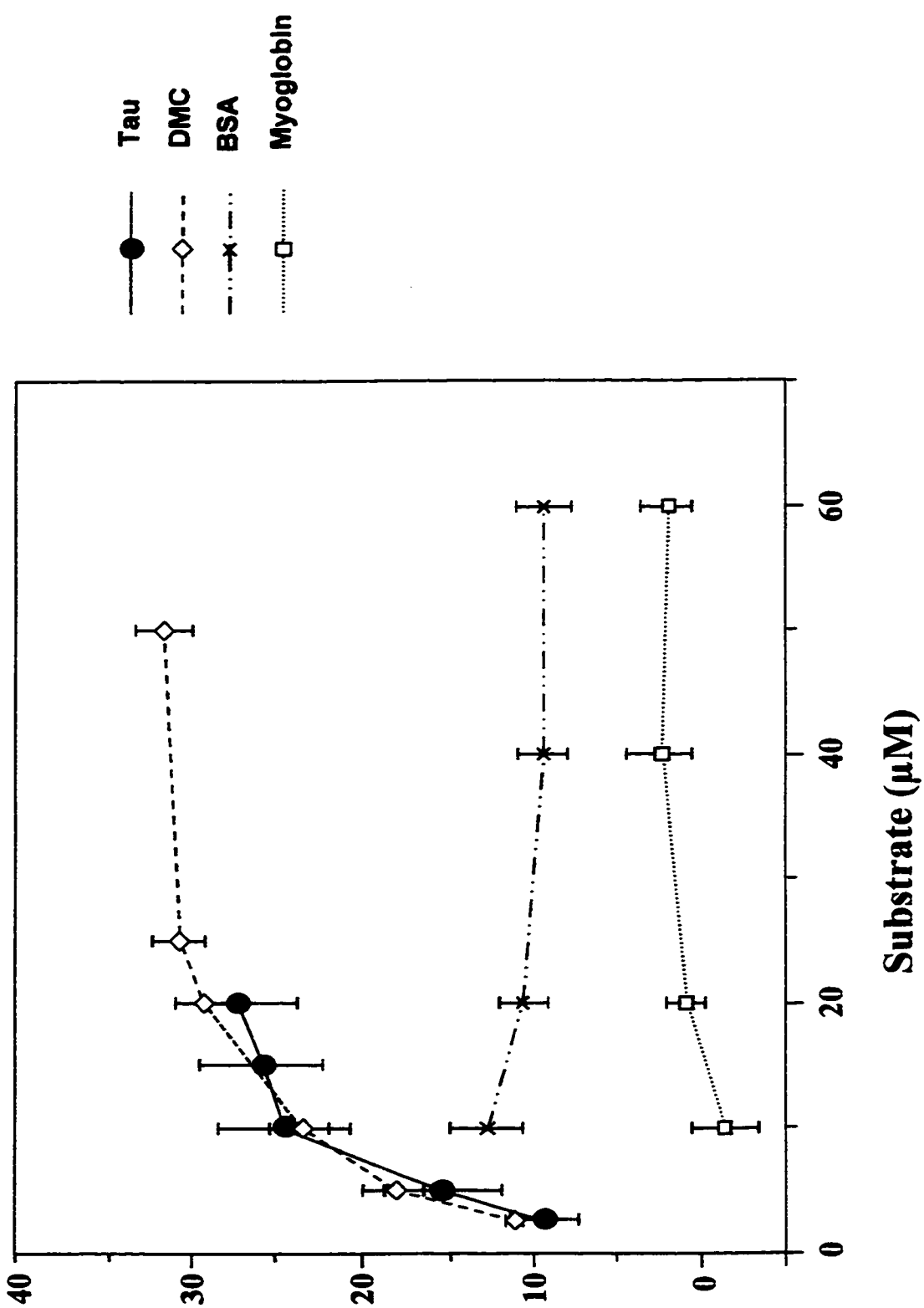
FIG. 2. High molecular weight "smearing" of τ in Alzheimer disease brain homogenates, and in bovine τ cross-linked with transglutaminase. Immunoblot of bovine τ incubated in the presence (−) or absence (-) of transglutaminase, and whole-homogenate samples of prefrontal cortex from control (C) and Alzheimer disease (AD) brains, probed with antibodies to τ. Bovine τ was incubated with or without tissue transglutaminase for 30 min, and aliquots containing 0.6 μg of τ were loaded onto an 8% polyacrylamide gel. Whole-homogenate samples of human prefrontal cortex in Laemmli sample buffer, from control (male, age 64 years) and Alzheimer disease (male, age 66 years) brains were loaded onto the same gel (30 μg of total protein). The resultant immunoblot was probed with an antibody cocktail containing Tau-2 (1:4,000), 7C11 (1:3,000), and 8C11 (1:10,000), as Tau-2 recognizes bovine τ but not human τ (Watanabe et al., 1992), and 7C11 and 8C11 recognize human τ selectively (Vigo-Pelfrey et al., 1995). The high molecular weight smearing, seen at ~100-150 kDa in the Alzheimer disease homogenate, is similar in appearance to bovine τ which has been cross-linked by transglutaminase.

To determine the extent to which τ is a substrate of transglutaminase, the incorporation of [^{3}H]-putrescine into purified bovine τ was measured. Initial measurements resulted in kinetic values for τ which were lower than expected. Further analysis revealed that a high degree of τ self-association resulted in transglutaminase-mediated cross-linking, a reaction which interfered with the incorporation of radiolabeled putrescine (see Fig. 1). To overcome this competing cross-linking reaction, the concentration of unlabeled putrescine was increased from 250 μM to 1 mM in the putrescine incorporation assay. Corresponding immunoblots indicate that most of the cross-linking is inhibited at this concentration of putrescine (data not shown). Complete inhibition of τ cross-linking by polyamines may not be possible, however, as samples incubated with concentrations of putrescine as high as 5 mM still contain detectable amounts of dimeric and polymeric τ (data not shown).

Increasing the concentration of putrescine from 250 μM to 1 mM greatly improved the kinetic data for τ. The amount of putrescine incorporated into τ (10μM) by transglutaminase more than doubled (from 0.249 ± 0.029 nmol at 250 μM, to 0.568 ± 0.048 nmol at 1 mM, $p < 0.001$) with this experimental modification. A plot of initial velocity versus substrate concentration for four potential transglutaminase substrates (Fig. 3) indicates that τ and DMC are both good substrates for transglutaminase and that myoglobin and BSA are not substrates. Thus, the transglutaminase-catalyzed incorporation of putrescine is limited to specific substrate proteins.

A Hanes-Woolf plot (Fig. 4) of data from the putrescine incorporation assays indicates that τ is indeed an excellent substrate of transglutaminase, having a K_m of 10.4 ± 2.2 μM, and a V_{max} of 40.9 ± 4.5 nmol/mg enzyme/min. These values are very similar to the values obtained for DMC (5.5 ± 0.5 μM and 35.8 ± 1.5 nmol/mg enzyme/min, Fig. 4), one of the best transglutaminase substrates identified to date (Facchiano et al., 1993).

FIG. 3. The transglutaminase-mediated incorporation of putrescine is a specific reaction. Plot of initial velocity versus substrate concentration data from the putrescine incorporation assay. Transglutaminase substrates, like bovine τ (•) and dimethylcasein (■), have long, linear stretches of amino acids that include at least one accessible glutamine residue (n = 6-7 separate experiments). BSA (x) and myoglobin (□) are primarily globular in nature and are not substrates of the enzyme (n = 3-4 separate experiments).



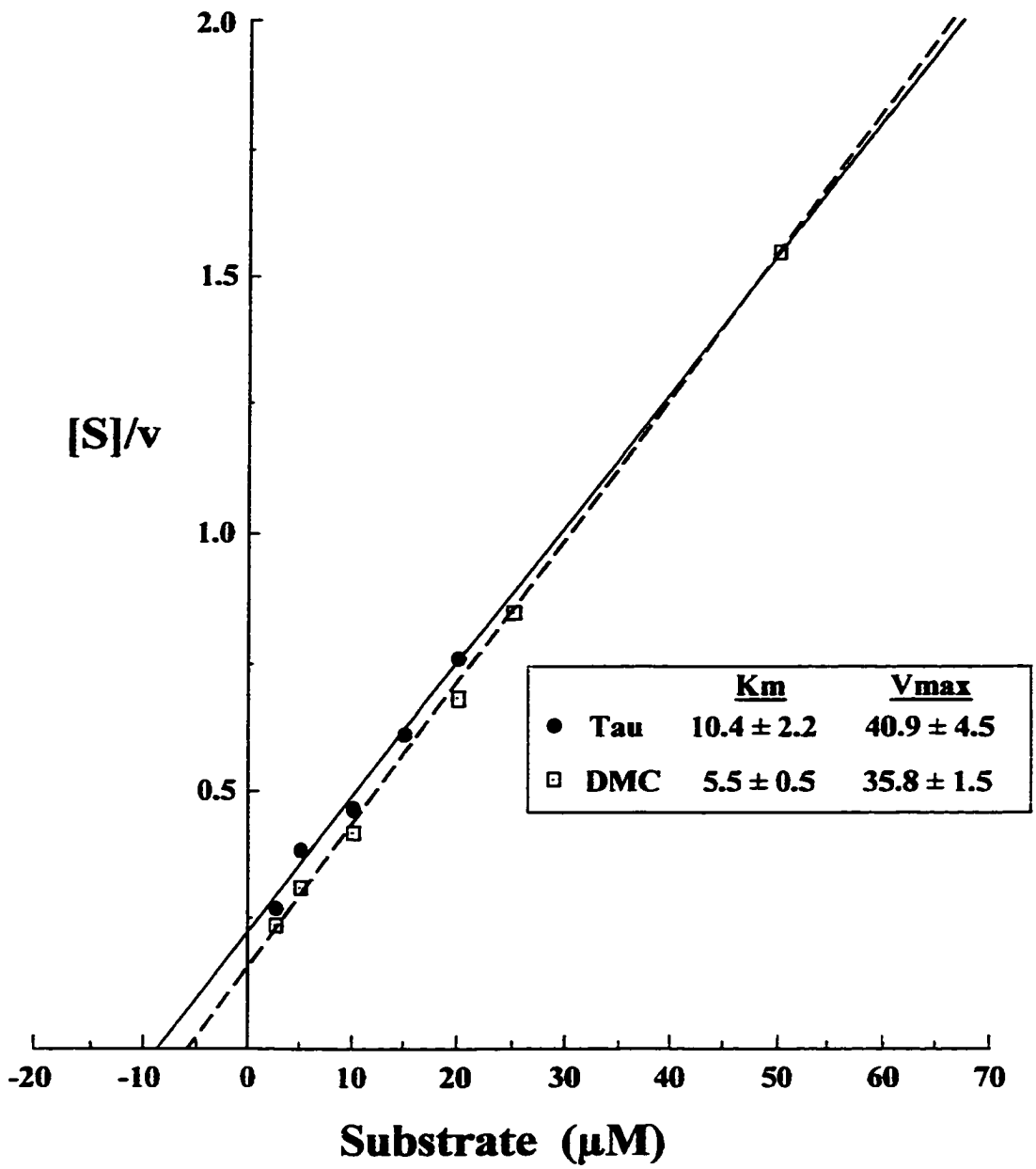


FIG. 4. τ is an excellent substrate for transglutaminase. Hanes-Woolf plot of data from the putrescine incorporation assay. Bovine τ is comparable to (DMC) as a substrate for transglutaminase (n = 6-7 separate experiments; representative data shown). Km values are micromolar; Vmax values are expressed as nanomoles per minute per milligram of transglutaminase.

τ isoform comparison

Because the isoforms of bovine τ appeared to be differentially cross-linked by transglutaminase (Dudek and Johnson, 1993), the substrate specificity of several recombinant human τ isoforms was examined using the [^{3}H]-putrescine incorporation assay described above. Isoforms T3, T4, and T4L were significantly better substrates for transglutaminase than purified bovine τ (Fig. 5). Furthermore, isoforms T4 and T4L, both of which contain four microtubule-binding domains were better substrates than T3, which only has three microtubule binding domains. In addition, the human τ construct T264, a 122 amino acid fragment of τ, was also a substrate of transglutaminase, although it was not as good a substrate as the isoforms or as purified bovine τ (Fig. 5). The stoichiometry of incorporation (moles of putrescine incorporated per mole of τ) for each isoform is indicated in the inset of Fig. 5.

Immunoblot analysis indicated that isoforms T3, T4, and T4L are all cross-linked by transglutaminase (Fig. 6A), as evidenced by the formation of dimers and of high molecular weight polymers that appear at the top of the gel. This is in contrast to construct T264, which apparently cannot be cross-linked by transglutaminase (Fig. 6B), even though it is a transglutaminase substrate in terms of putrescine incorporation (Fig. 5). A τ-immunoreactive band that migrates just ahead of each τ isoform is also evident in the samples which were incubated with transglutaminase (Fig. 6A). This immunoreactive band most likely represents either a cross-linked product of smaller τ fragments, or an intramolecular cross-linking of the isoform, which induces a conformational change that allows τ to migrate closer to its predicted molecular mass.

Proteolysis experiments

To determine which segments of the τ molecule were susceptible to modification by transglutaminase, the human τ isoforms T3 and T4 were radiolabeled with [^{3}H]putrescine, as described above. These isoforms were then partially proteolyzed by α-chymotrypsin,

FIG. 5. Transglutaminase differentially modifies the isoforms of human τ. Bar graph of data from the putrescine incorporation assay. Bovine τ, the recombinant human isoforms T3, T4, and T4L, and recombinant human τ deletion construct T264 (5 μM) and myoglobin (20 μM) were incubated with transglutaminase and [^{3}H]-putrescine as described in Experimental Procedures. T3 is a better substrate for transglutaminase than purified bovine τ (* $p < 0.05$), and T4 and T4L are better substrates than either bovine τ or T3 (** $p < 0.05$) (n = 5 separate experiments). Fragment T264 is also a transglutaminase substrate, although not as good a substrate as purified bovine τ († $p < 0.05$). Myoglobin is not a substrate of transglutaminase. (n = 5 for bovine τ and isoforms; n = 8 for T264; n = 4 for myoglobin.) **Inset:** The stoichiometry of putrescine incorporation (moles of putrescine incorporated per mole of τ).

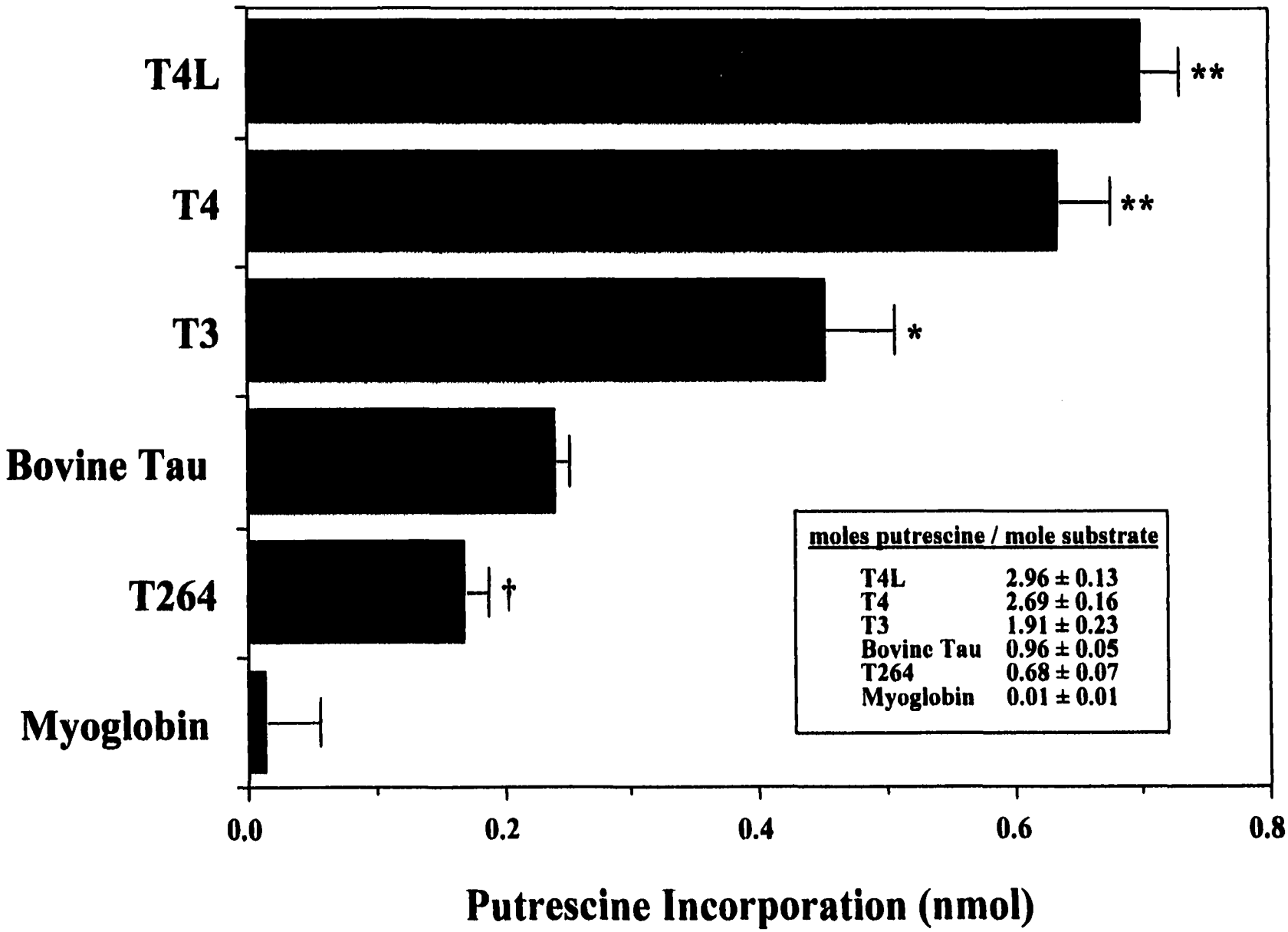
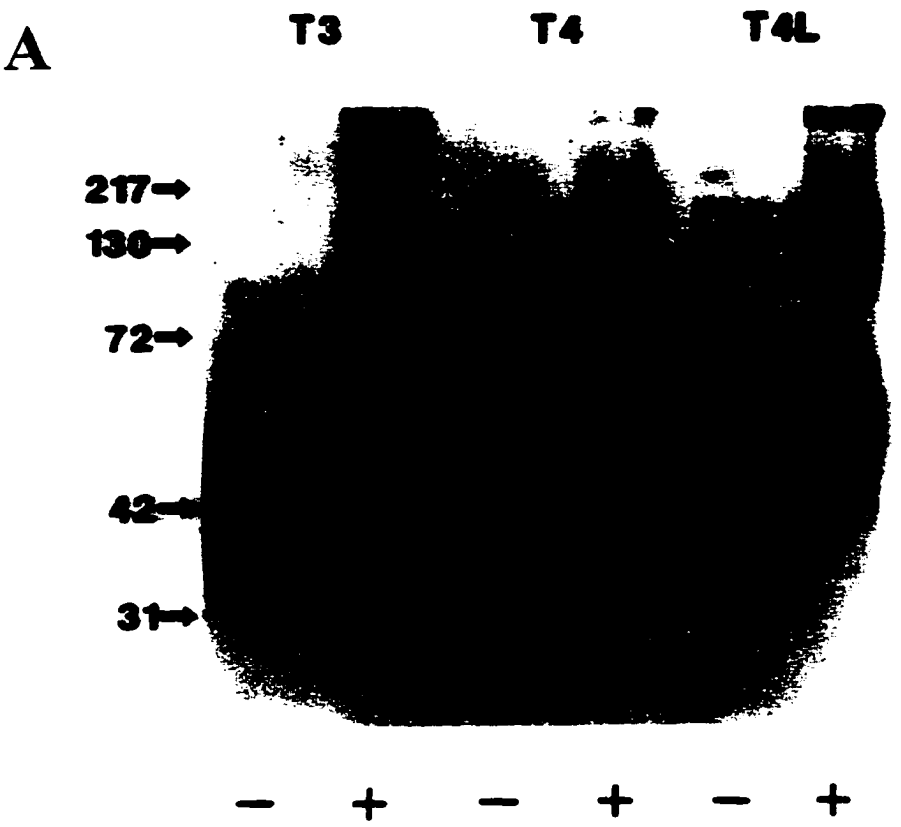
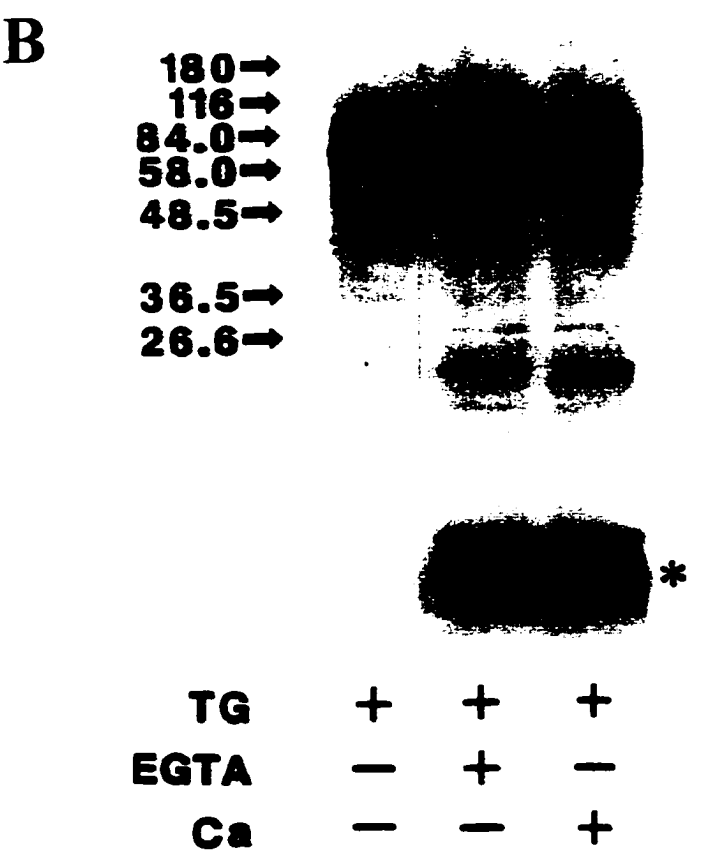


FIG. 6. Cross-linking of recombinant human τ isoforms by transglutaminase. **A:** Immunoblot (Tau-1, 1:12,000) of human τ isoforms T3, T4, and T4L (0.05 μg) incubated with transglutaminase in the presence of 5 mM EGTA(-) or 5 mM $CaCl_2$ (+) for 30 min at 37°C. All isoforms are cross-linked by transglutaminase to form high molecular weight multimers, including very high molecular weight polymers that are visible at the top of blot. **B:** Colloidal gold total protein stain of the recombinant human τ fragment T264 (*) incubated with transglutaminase (TG, shown by itself in the first lane) in the presence of 5 mM EGTA or 5 mM $CaCl_2$ (Ca) (as indicated) for 30 min at 37°C. T264 is not cross-linked by transglutaminase, as dimeric and higher molecular weight complexes cannot be detected. The two additional bands, not marked with an asterisk in the second and third lanes, are protein impurities from the bacterial expression system and are not immunoreactive with τ antibodies.



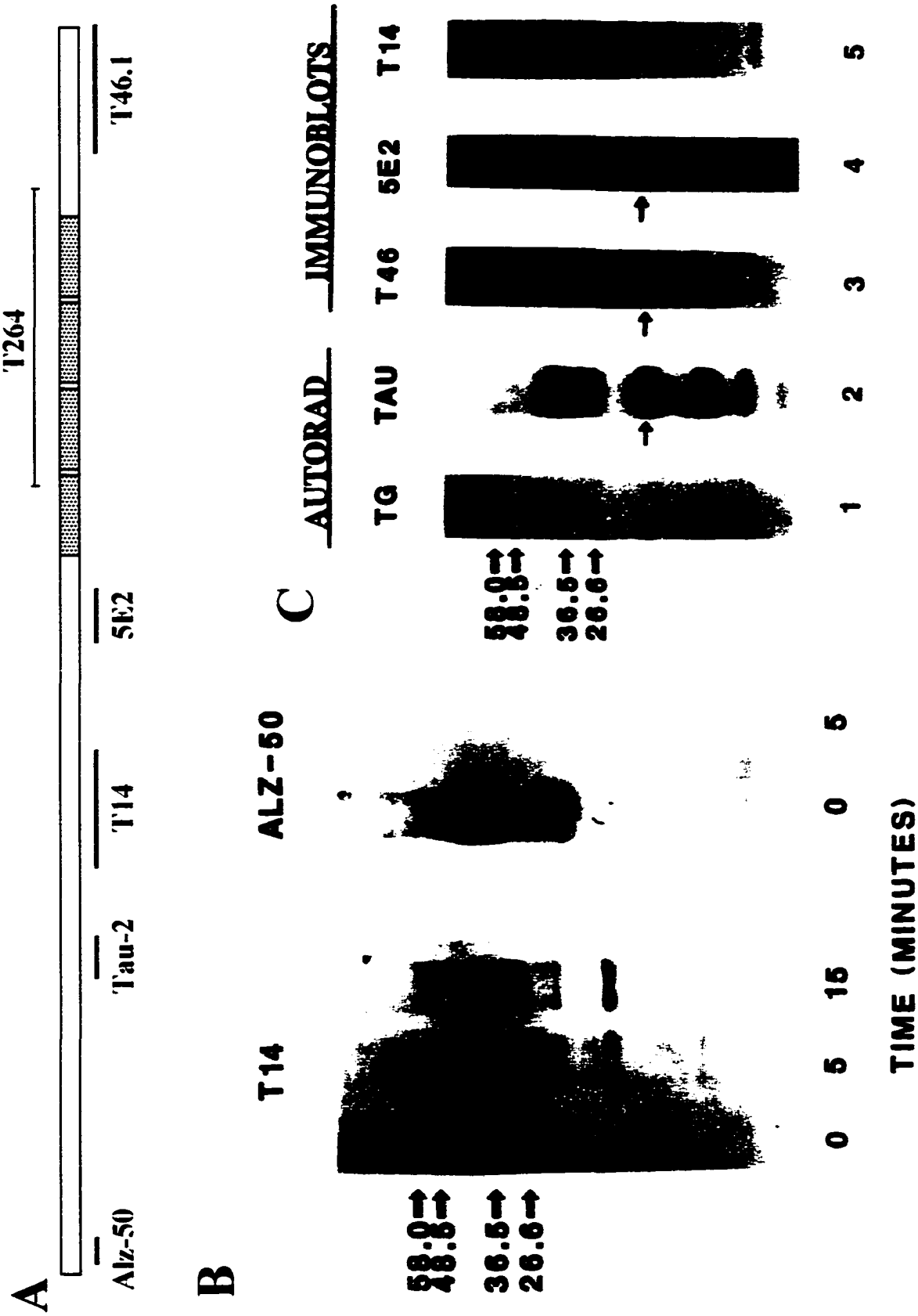


separated by electrophoresis, and examined by western blot analysis and autoradiography. To more clearly determine the cleavage sites of α-chymotrypsin, the same procedure was repeated with unlabeled putrescine, and the resulting proteolytic profile of T3 was determined by probing the blots with several different τ antibodies. The antibodies included Alz-50, τ-2, T14, 5E2, and T46.1, whose epitopes span the length of the τ molecule (Fig. 7A) (Kosik et al., 1988; Goedert et al., 1991*b*; Watanabe et al., 1992).

τ that has been modified by the addition of putrescine has an increased mobility on SDS-polyacrylamide gels compared to unmodified τ, and thus it migrates closer to the position expected from its predicted molecular mass (data not shown). Figure 7B shows the α-chymotrypsin proteolytic profile of putrescine-modified T3, as seen by immunoblotting with antibodies T14 and Alz-50. Complete cleavage occurs within 5 min of incubation with α-chymotrypsin, resulting in discrete breakdown products that remain stable throughout the entire 30-min proteolysis (Fig. 7B). One of the primary cleavage sites in T3 appears to be at a phenylalanine residue (Phe^{8}) in the middle of the Alz-50 epitope (Goedert et al., 1991*b*), because the immunoreactivity of this antibody is abolished within 5 min after the addition of the protease (Fig. 7B). However, as cleavage at this site alone is not enough to account for the increased mobility of the major τ band on SDS-polyacrylamide gels (compare lanes 1 and 2, Fig. 7B), it is likely that α-chymotrypsin also cleaves T3 at either Tyr^{18}, Tyr^{29}, or both, thus resulting in a 5-8% reduction in the size of T3 (see Fig. 7B).

After radiolabeling T3 with [^{3}H]-putrescine via the transglutaminase reaction, and proteolyzing with α-chymotrypsin, a comparison of the resulting autoradiographs and western blots revealed regions of the τ molecule that are potential sites of modification by transglutaminase. Even though the T3 isoform contains 14 glutamine residues that span the length of the molecule, only a few proteolytic fragments contain the radiolabel (Fig. 7C). The largest and most prevalent of these T3 fragments is a carboxy-terminal segment that contains the epitopes for T46.1 and 5E2 but not for T14 (Fig. 7C, arrows). This has been

FIG. 7. Proteolysis of putrescine-modified τ. **A:** Schematic diagram of the human T4L isoform, indicating the locations of antibody epitopes (underlined) (Kosik et al., 1988; Goedert et al., 1991; Watanabe et al., 1992). Also shown are the microtubule binding domains (shaded) and the location of the deletion construct T264. **B:** Immunoblots probed with antibodies T14 and Alz-50, showing the α-chymotrypsin proteolysis of putrescine-modified T3. Within 5 min of incubation with the protease, modified T3 is cleaved into a slightly lower-molecular weight form and several other fragments that are apparently not cleaved further (lanes 2 and 3). Production of the slightly lower-molecular weight fragment probably involves cleavage at the extreme amino-terminus of the protein, including a cleavage within the Alz-50 epitope, which abolishes the immunoreactivity of this antibody within 5 min of incubation with a-chymotrypsin (lane 5). See text for details. **C:** Proteolytic profiles of [^{3}H]-putrescine-labeled T3, visualized by autoradiography and immunoblot analysis. T3 was radiolabeled with [3H]-putrescine by transglutaminase as described in Experimental Procedures. This radiolabeled T3 was then proteolyzed by a-chymotrypsin for 30 min at 37°C. Autoradiographs of the sample indicate that transglutaminase (TG) is not labeled by this process (lane 1) and that only a few fragments of T3 (TAU) contain the radiolabel (lane 2). The major proteolytic fragment (lane 2, arrow), which contains the majority of the radiolabel, is also labeled by immunoblot analysis with antibodies T46.1 and 5E2 (lanes 3 and 4) but not with antibody T14 (lane 5), thus indicating a carboxy-terminal fragment (see A).



verified by antibody double-labeling experiments with T46.1, 5E2, and T14 (data not shown). This same fragment was also labeled in the T4 isoform, which has an extra microtubule binding domain in this region, and the resulting T4 fragment migrated slightly slower than the corresponding T3 fragment, as expected (data not shown). The other radiolabeled fragments of T3 were more difficult to identify; however, preliminary experiments involving the separation of radiolabeled τ fragments on a reverse-phase HPLC column indicate that one major proteolytic fragment and two secondary fragments contain most of the radiolabeled putrescine (unpublished data).

DISCUSSION

Tissue transglutaminase is an enzyme which can catalyze the cross-linking of substrate proteins into ordered, high molecular weight complexes that are rigid, insoluble, and resistant to proteolysis (Folk and Finlayson, 1977). This is of particular interest in Alzheimer's disease, as neurofibrillary tangles have biochemical properties that are similar to transglutaminase reaction products (Selkoe et al., 1982*b*). As mentioned earlier, the idea that transglutaminase is involved in neurofibrillary tangle formation is not a new one. Selkoe *et al.* (1982*a*;*b*) suggested that neurofibrillary tangles contain nondisulfide covalent bonds and proposed that transglutaminase was involved in the cross-linking of neurofilament proteins. Miller and Anderton (1986) also investigated the cross-linking of neurofilament and microtubule proteins and noted that the microtubule-associated proteins were "especially rapidly cross-linked." Given that the core of the neurofibrillary tangle consists of the τ protein (Kosik et al., 1986; Wood et al., 1986; Goedert et al., 1988; Lee et al., 1991), and that τ from Alzheimer disease brains presents as a smear similar to that observed when purified τ is cross-linked by transglutaminase (Fig. 2), we investigated the substrate properties of τ with respect to tissue transglutaminase. In this study, two separate but similar transglutaminase reactions were monitored, i.e., the incorporation of putrescine into substrate proteins and the cross-linking of substrate proteins (Fig. 1). Because cross-

linking by transglutaminase can only occur at glutamine residues that are modified by the enzyme, it is necessary to establish which glutamines can be modified by the enzyme (via the putrescine incorporation assay) before determining which of these sites are involved in the cross-linking of τ in vitro.

Purified bovine τ is an excellent substrate of transglutaminase, as determined by its putrescine incorporation (Fig. 3). Other proteins such as BSA and myoglobin are not substrates. The higher background seen in the BSA curve (Fig. 3) is probably due to a nonspecific precipitation of unincorporated [^{3}H]-putrescine rather than incorporation into the BSA protein itself, as the level of radiolabel precipitated with this protein does not change with increasing concentrations of BSA, up to 120 μM (data not shown). The human τ isoforms T3, T4, and T4L are even better substrates for transglutaminase than purified bovine τ (Fig. 5). Although the reason for these differences is unknown, several factors could be involved, including the following: (1) T3, T4, and T4L are purified constructs, while the bovine τ contains all isoforms, several breakdown products, and possible impurities which may add to the protein concentration without being modified by transglutaminase; (2) sequence differences between human and bovine τ may make human τ an inherently better substrate of transglutaminase than bovine τ; and (3) the isoforms are bacterially expressed and thus do not contain posttranslational modifications, which may or may not alter putrescine incorporation. In a comparison of the isoforms, T4 and T4L, both of which contain four microtubule binding domains, are better substrates than T3 (Fig. 5), which has only three microtubule binding domains. This may be because the four-repeat isoforms are in a conformation that is more accessible to transglutaminase or because they contain one extra glutamine that may be modified by the enzyme.

The human τ construct T264 is also a substrate of transglutaminase, albeit not as good a substrate as the isoforms or as purified bovine τ (Fig. 5). However, although T264 is a substrate of transglutaminase, it cannot be cross-linked by the enzyme (Fig. 6B). This appeared somewhat surprising at first, especially considering that T264 contains 16 lysine

residues that could potentially react with the enzyme-substrate intermediate. However, this finding agrees with studies indicating that transglutaminase is remarkably selective for the lysines it utilizes in cross-linking reactions (Parameswaran et al., 1990; Lorand et al., 1992). Thus T264 provided an excellent control, demonstrating that putrescine incorporation and cross-linking are indeed two distinct reactions; i.e., transglutaminase is able to incorporate putrescine into all of its substrates; however, only certain substrates can be cross-linked by the enzyme. It can therefore be postulated that to be cross-linked by transglutaminase, substrates must first be oriented in a specific conformation, wherein the lysine residue of the "donor" protein is closely apposed to the substrate glutamine residue of the "acceptor" protein. Such an orientation is needed to ensure that the lysine is incorporated rather than another primary amine. If this is the case, then only substrate proteins that are already "self-associated" in a specific arrangement would have the potential to be cross-linked by transglutaminase. In agreement with this hypothesis, there is evidence to suggest that τ readily self-associates into dimers and higher order oligomers and can form soluble filamentous structures (Wille et al., 1992; de Ancos et al., 1993; Crowther et al., 1994), a process that requires the presence of the microtubule-binding domains of τ (Wischik et al., 1988; Jakes et al., 1991; Ksiezak-Reding and Yen, 1991; Wille et al., 1992). Even under reducing conditions and in the absence of transglutaminase, a small amount of τ can often be seen in the dimeric form on an immunoblot (data not shown). It is possible that this self-aggregation requires the first microtubule binding domain of τ, and as construct T264 lacks most of the first microtubule binding domain, this could explain why it is not cross-linked by transglutaminase (Fig. 6B). It must be noted, however, that even a protein that aggregates, but that does not have a lysine next to a substrate glutamine residue, will not likely be cross-linked by transglutaminase, and this possibility also exists for T264.

Predicting the ability of a given protein to be modified by transglutaminase, based on primary amino acid sequence, is not straightforward. Neither the absolute number of

glutamines, which are involved in the first catalytic step of the transglutaminase mechanism (Folk and Finlayson, 1977; Greenberg et al., 1991), nor the percentage of glutamines in a protein, appears to be sufficient to qualify a protein as a transglutaminase substrate. Based on the number of glutamine residues alone, it would be expected that T4L is a better substrate than T4; however, these isoforms are comparable substrates. Myoglobin contains five glutamine residues, but it is not a substrate. The amino acid sequence of T264 consists of 4.9% glutamine residues, whereas the T4L sequence contains only 4.3%; yet T4L is a much better substrate than T264. Thus, because not all glutamine residues within a substrate protein can be modified by transglutaminase, the differences in glutamine compositions among the isoforms are not sufficient to account for their differences in modification by the enzyme. To further complicate matters, there appears to be no particular primary amino acid consensus sequence for transglutaminase, although a glutamine residue that is modified by the enzyme is usually positioned in either a highly flexible part of the protein or in a sequence of linear secondary structure, thereby making it accessible to the enzyme (Coussons et al., 1992). Also, certain charged amino acid residues located near the glutamine may enhance or inhibit binding (Coussons et al., 1992; Facchiano and Luini, 1992).

To determine the relative locations of the glutamine residues in τ that are modified by transglutaminase, T3 and T4 were labeled with [^{3}H]-putrescine by transglutaminase, proteolyzed with α-chymotrypsin, and the breakdown products were analyzed by auto-radiography and immunoblotting. Results of these experiments indicate that trans-glutaminase selectively modifies only one or a few of the numerous glutamine residues in the τ sequence. Only three proteolytic fragments of T3, and only two to three fragments of T4, contained label (Fig. 7C and unpublished observations). The primary radiolabeled fragment from T3 is a carboxy-terminal fragment which contains the epitopes for T46.1 and 5E2 (Fig. 7C, arrows), although the number of modified glutamines in this fragment could not be ascertained by our methods. It is speculated that this fragment represents a

carboxyl segment of T3 that has been cleaved at Tyr^{197}. Such a cleavage would generate a carboxy terminal breakdown product with an approximate molecular mass of 23,300 Da, which would include epitopes for T46.1 and 5E2 but not T14. This agrees with theories that the PHF backbone consists of τ arranged in an anti-parallel fashion, joined somewhere near the carboxyl end of the molecule (Kondo et al., 1988; Wischik et al., 1988; Ksiezak-Reding and Yen, 1991; Wille et al., 1992). The other two radiolabeled fragments of T3 were more difficult to identify through western blotting techniques; however, preliminary evidence from HPLC separation of the proteolytic fragments of T3 indicates that most of the radiolabeled peptides elute as one major and two secondary peaks (unpublished data).

It should be noted that transglutaminase is a calcium-activated enzyme (Folk and Finlayson, 1977). Much evidence suggests that a disruption in calcium homeostasis leads to increased cytosolic calcium in Alzheimer disease (for review, see Mattson, 1992). For example, increased levels of calcium-binding proteins (Hof et al., 1991; Sutherland et al., 1993) and activated calpain (Saito et al., 1993) have been noted in Alzheimer disease brains, and increased concentrations of bound, cytosolic, and free calcium have been found in fibroblasts from Alzheimer patients (Peterson and Goldman, 1986). Studies by Mattson (1992) have shown that sustained or increased levels of intracellular calcium result in the depolymerization of microtubules in cultured cortical neurons. It has been proposed that this depolymerization of microtubules may lead to an increase in the concentration of free, unbound τ, which is then available to self-aggregate (Mattson, 1992). Because transglutaminase is also activated by calcium, pathological increases in calcium could provide a mechanism whereby this aggregated τ could be exposed to abnormal transglutaminase activity. It is interesting that Mattson (1992) noted that calcium influx led not only to the depolymerization of microtubules, but also to the formation of 8-15-nm straight filaments and an increased immunoreactivity with the Alz-50 antibody. Increased Alz-50 immunoreactivity is also seen when τ is cross-linked by transglutaminase in vitro (Dudek and Johnson, 1993).

Because of the unique properties of transglutaminase activation and catalysis, and the characteristics of its interaction with τ, it can perhaps be hypothesized that transglutaminase, or some similar cross-linking enzyme, may be involved in converting τ from its normal, soluble form to a polymerized, insoluble form, such as that found in the neurofibrillary tangle. If soluble PHFs, which contain τ in an ordered, antiparallel state (Ksiezak-Reding and Yen, 1991; Wille et al., 1992), were pathologically exposed to increased calcium levels and abnormal transglutaminase activity, the result could be ordered, insoluble aggregations of τ (Dudek and Johnson, 1993) such as that seen in neurofibrillary tangles. This type of stabilization is not unprecedented, as it occurs in transglutaminase-mediated clot formation; wherein fibrin polymers that are initially held together by noncovalent bonds become stabilized by ε-(γ-glutamyl)lysine bonds (Folk and Finlayson, 1977). A similar reaction may occur in the keratinization of the epidermis, where soluble epidermal structural proteins are cross-linked into insoluble high molecular weight polymers (Folk, 1980).

In summary, τ is an excellent in vitro substrate of tissue transglutaminase, comparable with DMC, one of the best substrates known (Facchiano et al., 1993). The human τ isoforms are differentially cross-linked, as T4 and T4L are better transglutaminase substrates than T3. In the absence of polyamines, transglutaminase cross-links τ into dimers, trimers, and high molecular weight aggregates. This cross-linking of τ is a specific reaction, as a partial segment of τ (T264), which lacks part of the first microtubule-binding domain, is a substrate for transglutaminase but cannot be cross-linked. Because transglutaminase-cross-linked proteins contain ε-(γ-glutamyl)lysine bonds rather than disulfide bonds, transglutaminase-cross-linked τ is insoluble in detergents and reducing agents. This may be important in pathological conditions such as Alzheimer disease, in which τ has been shown to form aggregated, insoluble polymers. τ is modified by transglutaminase at only one or a few specific sites, including at least one site in the carboxyl half of the

molecule. This agrees with previous findings that the cores of soluble PHFs consist of the carboxyl 40% of the τ molecule (Ksiezak-Reding and Yen, 1991).

It must be emphasized, however, that there is as yet no direct evidence for the existence of ε-(γ-glutamyl)lysine isopeptide bonds in neurofibrillary tangles. As noted previously (Selkoe et al., 1982*a*), attempts to detect such bonds should be highly informative. Further research is required to elucidate the putative role of transglutaminase in the formation of neurofibrillary tangles in Alzheimer disease. However, even if transglutaminase itself is not involved in the formation of neurofibrillary tangles, it provides a useful model system for examining the properties of aggregated, insoluble τ polymers, which are a hallmark of Alzheimer disease.

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TRANSGLUTAMINASE ACTIVITY IS INCREASED IN ALZHEIMER'S DISEASE BRAIN

by

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ABSTRACT

Transglutaminase is a calcium-activated enzyme that cross-links substrate proteins into insoluble, often filamentous aggregates resistant to proteases. Because the neurofibrillary tangles in Alzheimer's disease have similar characteristics, and because τ protein, the major component of these tangles is an excellent substrate of transglutaminase in vitro, transglutaminase activity and levels were measured in control and Alzheimer's disease brain. Frozen prefrontal cortex and cerebellum samples from Alzheimer's disease and control cases matched for age and postmortem interval were used in the analyses. Total transglutaminase activity was significantly higher in the Alzheimer's disease prefrontal cortex compared to control. In addition the levels of tissue transglutaminase, as determined by quantitative immunoblotting, were elevated approximately 3-fold in Alzheimer's disease prefrontal cortex compared to control. To our knowledge, this is the first demonstration that transglutaminase is increased in Alzheimer's disease brain. There were no significant differences in transglutaminase activity or levels in the cerebellum between control and Alzheimer's disease cases. Because the elevation of transglutaminase in Alzheimer's disease sample occurred in the prefrontal cortex, where neurofibrillary pathology is usually abundant, and not in the cerebellum, which is usually spared in Alzheimer's disease, it can be suggested that transglutaminase could be a contributing factor in neurofibrillary tangle formation.

INTRODUCTION

A defining hallmark of Alzheimer's disease pathology is the presence of numerous neurofibrillary tangles in the neocortex and hippocampus (Braak and Braak, 1991), lesions that are rarely if ever present in the cerebellum (Dustin et al., 1992). Neurofibrillary tangles are composed primarily of paired helical filaments (PHFs) and are extremely insoluble structures (Selkoe et al., 1982*b*). The insoluble PHFs of the neurofibrillary tangles, like the soluble PHFs, are formed from the microtubule-associated phosphoprotein

τ (Kosik et al., 1986; Wood et al., 1986). In addition, the majority of τ which forms the PHFs is extensively hyperphosphorylated compared to τ not associated with these structures (Lee et al., 1991; Goedert, 1993; Morishima-Kawashima et al., 1995). It has been hypothesized that τ first aggregates as soluble PHFs which are subsequently converted by covalently cross-linking to form the insoluble PHFs of the neurofibrillary tangles (Crowther et al., 1992; Goedert et al., 1992; Goedert, 1993). The critical processes involved in stabilizing τ aggregation and converting soluble PHFs into insoluble structures are as of yet unknown.

One mechanism which could contribute to the aggregation of τ and the production of the insoluble neurofibrillary tangles is the catalysis of covalent cross-links between τ molecules by transglutaminase. The transglutaminases are a family of calcium-activated enzymes that catalyze the formation of ε-(γ-glutamyl)lysine isopeptide bonds between substrate proteins rendering the resulting cross-linked protein complexes insoluble (Folk and Finlayson, 1977; Greenberg et al., 1991). Specific transglutaminases are involved in a wide variety of processes including the cornification of skin and cross-linking of fibrin in clot formation (for a review, see Greenberg et al., 1991). In addition, early studies demonstrated the presence of transglutaminase activity in human brain (Selkoe et al., 1982*a*). The transglutaminase family member, tissue transglutaminase, which is primarily a cytosolic enzyme, has been immunocytochemically localized within neurons (Perry et al., 1995) and is abundant in nerve terminals in rat brain (Gilad and Varon, 1985). Tissue transglutaminase is an inducible enzyme (Gilad and Varon, 1985; Maccioni and Seeds, 1986) and has been shown to be upregulated during apoptosis (Fesus et al., 1987, 1989; El Alaoui et al., 1992). This is intriguing because recent evidence indicates that apoptosis may be involved in the neurodegeneration associated with Alzheimer's disease (Cotman and Anderson, 1995).

τ protein was recently identified as an excellent in vitro substrate of tissue transglutaminase (Miller and Johnson, 1995), and filaments were formed by the cross-linked τ

(Dudek and Johnson, 1993). Because transglutaminase catalyzes the formation of iso-peptide bonds between substrate proteins, the cross-linked filamentous aggregates of τ were insoluble, suggesting that this may be a contributing step in the process leading to the formation of neurofibrillary tangles. The hypothesis that transglutaminase could be involved in the formation of insoluble neurofibrillary tangles is not a novel one, but was investigated more than ten years ago (Selkoe et al., 1982*a*,*b*). However, those studies predated the discovery of τ as the major protein constituent of the PHFs, and, therefore focused primarily on neurofilaments as potential transglutaminase substrates (Selkoe et al., 1982*a*,*b*; Miller and Anderton, 1986).

Tissue transglutaminase is a dual function protein so that besides covalently cross-linking proteins, it also functions as a signal transducing G protein (Nakaoka et al., 1994). Tissue transglutaminase binds GTP and possesses intrinsic GTPase activity, as do typical G-proteins (Lee et al., 1989). In vitro, GTP significantly inhibits the transglutaminase activity of the enzyme (Achyuthan and Greenberg, 1987) and converts it to a signal-transducing G-protein called Gh, which activates the δ-subtype of phospholipase C (Nakaoka et al., 1994; Hwang et al., 1995). In COS-1 cells transfected with tissue transglutaminase, transglutamination activity is inhibited by GTPγS or by α-adrenergic receptor activation, and there is a concomitant increase in its activation of phospholipase C (Nakaoka et al., 1994). Shimohama and colleagues have demonstrated that the specific activity of phospholipase C-δ is significantly decreased in Alzheimer's disease brain (Shimohama et al., 1995), and phospholipase C-δ antibodies immunostain neurofibrillary tangles, dystrophic neurites, and neuropil threads (Shimohama et al., 1991). These pathological structures were not immunostained by antibodies to phospholipases ß-1, γ-1, and γ-2 (Shimohama et al., 1991).

If transglutaminase is involved in τ aggregation and neurofibrillary tangle formation, it can by hypothesized that transglutaminase activity and/or expression may be elevated in Alzheimer's disease. Therefore, total transglutaminase activity and the levels of

tissue transglutaminase were measured in samples from prefrontal cortex and cerebellum of control and Alzheimer's disease cases. When the samples were matched for age and postmortem interval, transglutaminase activity and levels were found to be increased significantly in Alzheimer's disease prefrontal cortex compared to controls. No differences in the levels or activity of transglutaminase were observed in the cerebellum.

MATERIALS AND METHODS

Tissue

Frozen human prefrontal cortex and cerebellum samples, which had been coded to protect the identities of the subjects, were obtained from the University of Alabama at Birmingham Brain Resource Program, directed by R.E.P. All patients with Alzheimer's disease met NINCDS/CERAD criteria, and all control subjects were free of neurological disease. Patients with mixed pathology, e.g., Alzheimer's and diffuse Lewy body disease, were excluded from the study.

Transglutaminase assay

Transglutaminase activity was measured in samples from prefrontal cortex and cerebellum using a modification of the procedure of Hand et al. (1993*a*). Transglutaminases react with free glutamine residues in substrate proteins, releasing ammonia, and then the enzyme-substrate intermediate reacts with an appropriate, nearby primary amine. This can either be the ε-amino group of lysine on an adjacent protein, resulting in cross-linking, or the primary amino group of a polyamine such as putrescine, resulting in polyamine incorporation into the substrate (Greenberg et al., 1991). In this assay, the incorporation of [3H] putrescine into the protein was used as a measure of transglutaminase activity. Tissue samples (~100 mg wet weight) were homogenized (1:10, w/v) in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A. A particle-free

supernatant was prepared from the homogenate by centrifugation at 16,000 x g for 10 min at 4°C. The assay buffer contained 10 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 5 mM dithiothreitol (DTT), 15 mM NaCl, 0.2 mM putrescine (unlabeled), 1 μCi [1,4 (n)-3H] putrescine dihydrochloride (Amersham International, Inc., Arlington Heights, IL, U.S. A.), 5 mM $CaCl_2$, 3 mg/ml N,N, - dimethylated casein (DMC), protease inhibitors, and 50 μl of tissue supernatant (total assay volume, 100 μl). Background values were obtained by incubation of the tissue supernatant in a buffer which contained 5 mM EGTA with no $CaCl_2$ or DMC. All measurements were done in triplicate. Samples were incubated for one h at 37°C, and the reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 8.5%. The samples were incubated on ice for 60 min and then centrifuged for 20 min at 16,000 x g at 4°C. The supernatant was removed, the pellet was rinsed twice with 5% TCA, and 250 μl of 0.25 N NaOH was added to each tube. The samples were incubated in a boiling water bath for 5 min, cooled and mixed vigorously. Aliquots (200 μl) of each sample were removed, and [^{3}H]-putrescine incorporation into the precipitated proteins was determined by liquid scintillation counting. Protein concentrations were determined by the method of Lowry et al. (1951) and transglutaminase activity was calculated after background subtraction as pmol putrescine incorporated/h/mg protein.

Transglutaminase antibodies

The tissue transglutaminase antibody CUB 7402 was a generous gift from Dr. P. Birckbichler (Birckbichler et al., 1985). The monoclonal tissue transglutaminase antibody 4C1 was prepared using BALB/c mice by the hybridoma core facility at The University of Alabama at Birmingham. The immunogen was tissue transglutaminase which was purified from a crude preparation supplied by the Sigma Chemical Co. (St. Louis, MO, U.S.A.) using the procedure described by Lee et al. (1989). Screening of positive clones was done by ELISA and immunoblot analysis. Undiluted tissue culture media from the 4C1 clones was used as the source of the antibody.

Immunoblotting

Tissue samples (~100 mg wet weight) from prefrontal cortex and cerebellum were homogenized (1:5, w/v) in 250 mM Tris-Cl, pH 6.8, 2% sodium dodecylsulfate (SDS), 25 mM DTT, 5 mM EGTA, 5 mM EDTA, and 10% glycerol. Samples were incubated in a boiling water bath for 5 min and clarified by centrifugation. Protein concentrations were determined by the method of Lowry et al. (1951) after acid precipitation of the protein. Bromophenol blue was added to the samples prior to electrophoresis. Samples (100 μg) were resolved on 8% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were blocked for 1 h at room temperature in 5% nonfat dry milk, 0.2% NP40 in borate saline. The blots were then incubated in the same buffer for 1 h with antibody (1-2 μg/ml). Blots were rinsed once with 2% NP40, 0.2% SDS in borate saline and incubated with horseradish peroxidase-conjugated goat antimouse IgG (1:3000) in this buffer for 1 h. The blots were then rinsed 3 x 5 min with 2% NP40, 0.2% SDS in borate saline, followed by four quick rinses with distilled water and developed with enhanced chemiluminescence (Amersham International, Inc.) following the manufacturer's instructions. The immunoblots obtained in this manner resulted in values that were in the linear response range. All data were analyzed using a BioRad GS-670 imaging densitometer and normalized to an internal standard to eliminate blot to blot variations in staining intensity.

Statistics

Data were evaluated using the paired *t* test, and values were considered significantly different when the two-tailed *p* value was less than 0.05.

RESULTS

Transglutaminase activity

In this study transglutaminase activity was measured in the prefrontal cortex and cerebellum of nine Alzheimer's disease cases and nine controls matched for age and

postmortem interval (PMI). The demographics of the nine control and matched Alzheimer's cases are shown in Table 1. The average age of the controls was 70.4 + 2.4 years, and the average of the Alzheimer's cases was 70.6 + 2.3 years. The PMI's of the control and Alzheimer's cases were 7.9 + 1.6 and 7.6 + 1.4 h, respectively. In the

TABLE 1

Demographic details of subjects studied

Sample	Sex	Age (yrs)	PMI (h)[a]	Cause of death
Control	F	69	5	Gram negative septicemia
Alzheimer's	M	66	5	Unknown
Control	M	64	10	Respiratory failure
Alzheimer's	M	67	8	Systemic infection
Control	F	80	6	Cardiac arrest
Alzheimer's	F	78	6	Respiratory failure
Control	M	74	5	Respiratory failure
Alzheimer's	F	74	3	Pneumonia
Control	M	77	5	Congestive heart failure
Alzheimer's	F	78	4	Renal failure
Control	M	63	6	Myocardial infarction
Alzheimer's	M	64	5	Myocardial infarction
Control	M	77	16	Coronary heart disease
Alzheimer's	M	78	15	Pneumonia
Control	F	73	16	Cardiac arrest
Alzheimer's	M	73	15	Respiratory failure
Control	M	57	2	Cardiac arrest
Alzheimer's	F	57	7	Unknown

[a] Postmortem interval (PMI) was defined as the interval between the time of death and flash-freezing of brain samples.

prefrontal cortex of the Alzheimer's disease specimens, the transglutaminase activity was 525 + 62 pmol putrescine incorporated/h/mg protein, which was significantly greater than the activity in the control samples (402 + 63 pmol putrescine incorporated/h/mg protein) (Fig. 1). In only one of the nine matched sets was the transglutaminase activity in the prefrontal cortex lower in the Alzheimer's case compared to the control (Fig. 1). In contrast, there were no significant differences in transglutaminase activity in the cerebellum

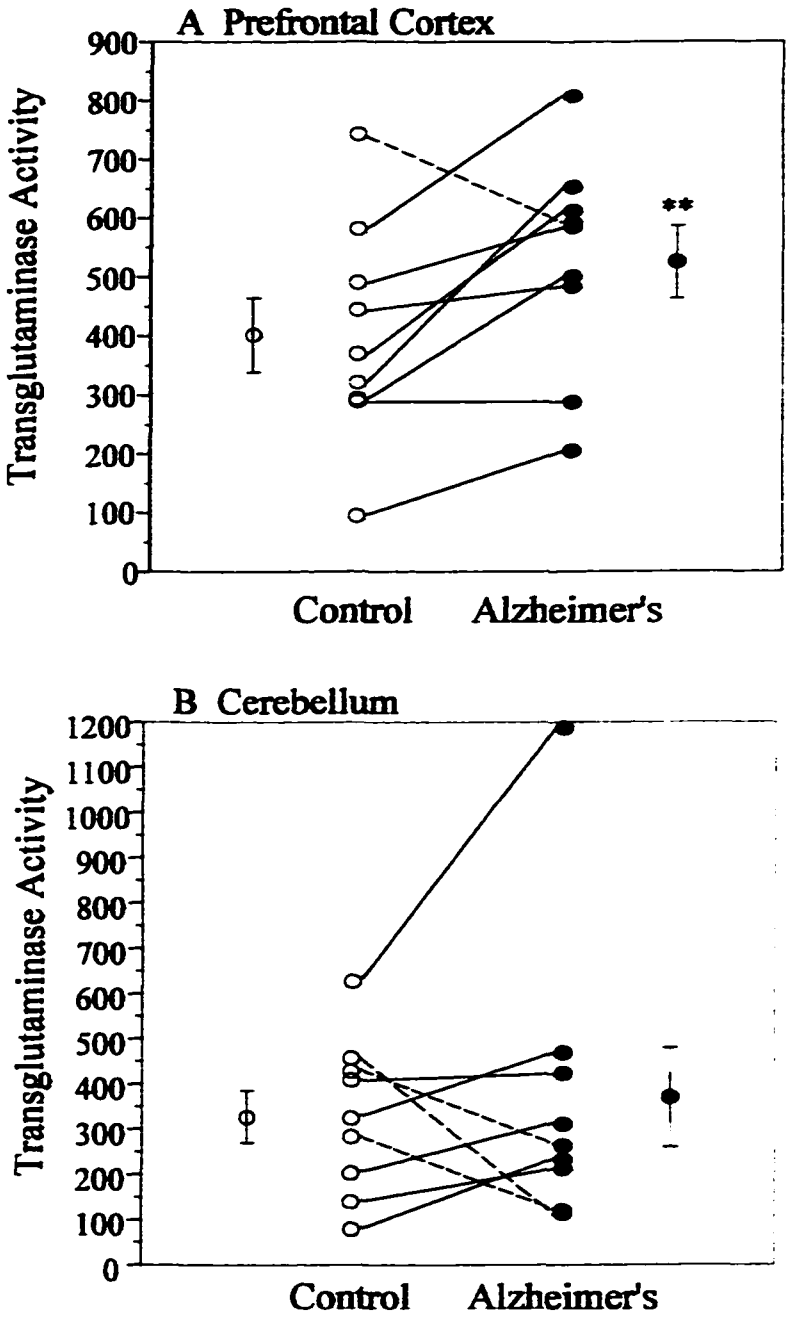

FIG. 1. Transglutaminase activity (pmol putrescine incorporated/hr/mg protein) in the prefrontal cortex (**A**) and cerebellum (**B**) of control (o) and Alzheimer's disease (•) cases. Nine controls and Alzheimer's cases were paired on the basis of age and PMI. In only a single case was transglutaminase activity lower in the Alzheimer's prefrontal cortex compared to its age- and PMI-matched control, in contrast to 3 out of the 9 in the cerebellum (dashed lines signify transglutaminase activity values that were lower in the Alzheimer's disease case compared to the control). Symbols with error bars signify mean ± SEM. ** $p < 0.05$.

of control and Alzheimer's disease cases (activities were 328 + 54 and 370 + 109 pmol putrescine/h/mg protein for control and Alzheimer's disease samples, respectively) (Fig. 1). In the cerebellum, transglutaminase activity was significantly higher in both the control and Alzheimer's disease samples of one matched set of samples compared to the other eight sets. It is unclear why the activity in both the control and Alzheimer's disease samples of this set were elevated, given their demographics and the fact that the causes of death were not the same (the control died of lung cancer and the Alzheimer's disease case died of systemic infection). However, even if this set was not included in the statistical analysis, there was still no significant difference in transglutaminase activity in the cerebellum between control and Alzheimer's disease cases.

Tissue transglutaminase levels

To determine the levels of tissue transglutaminase, samples from the prefrontal cortex and the cerebellum of the nine matched control and Alzheimer's disease cases were quantitatively immunoblotted. To establish that 4C1 was recognizing tissue transglutaminase specifically, identical samples from prefrontal cortex were probed with the well-characterized tissue transglutaminase monoclonal antibody CUB 7402 (Birckbichler et al., 1985) or the monoclonal antibody 4C1. Both antibodies recognized identical bands at approximately 78 kDa (Hand et al., 1993*b*; Ohashi et al., 1995) on the immunoblots (Fig. 2), validating the ability of 4C1 to selectively recognize tissue transglutaminase. Representative tissue transglutaminase immunoblots of samples from the prefrontal cortex and cerebellum of control and Alzheimer's disease specimens are shown in Figure 3. Quantitative analysis of immunoblots from the nine matched cases is shown in Figure 4. The levels of tissue transglutaminase in the prefrontal cortex were significantly elevated almost three-fold in Alzheimer's disease compared to the controls. As with transglutaminase activity, there were no significant differences in the levels of the enzyme in control and Alzheimer's disease cerebellum.

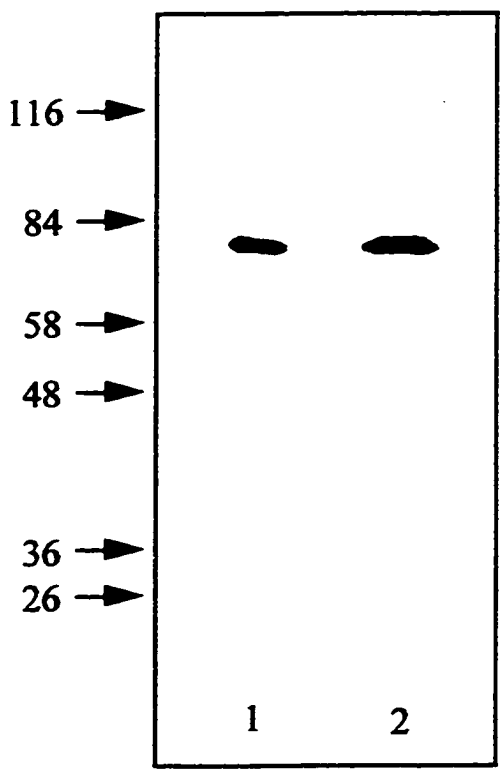

FIG. 2. Homogenates (100 μg) of the prefrontal cortex from a control were run in each lane of an 8% SDS-polyacrylamide gel and immunoblotted with tissue transglutaminase monoclonal antibodies 4C1 (lane 1) or CUB 7402 (lane 2). Both antibodies recognize identical bands at approximately 78 kDa.

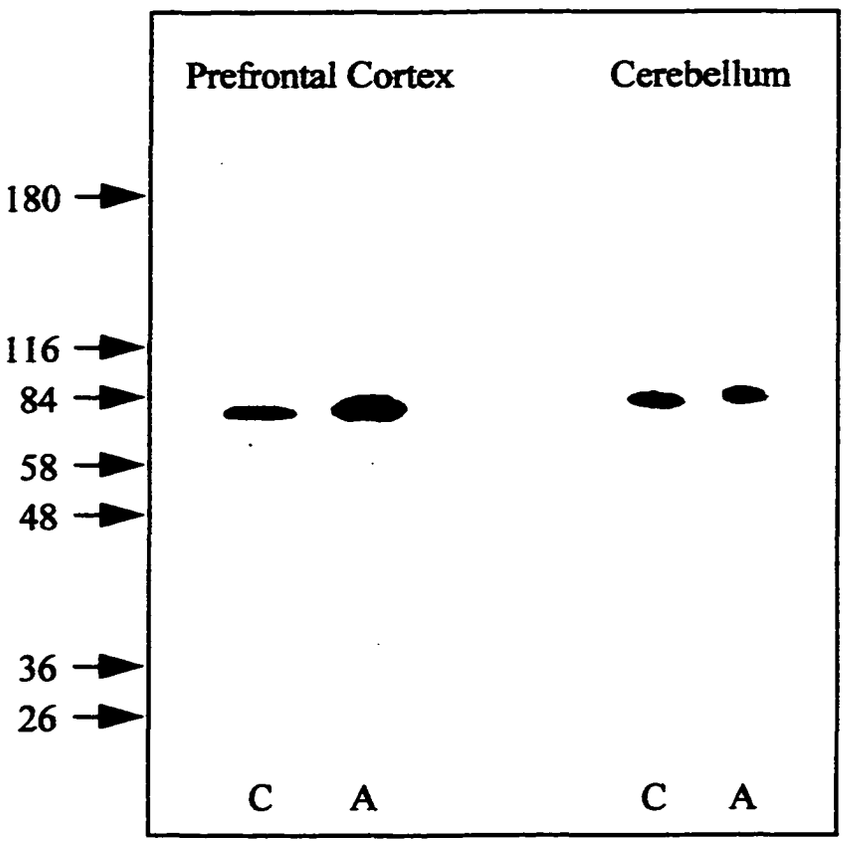

FIG. 3. Representative immunoblots of a control (C) and matched Alzheimer's disease (A) sample (100 μg) probed with the tissue transglutaminase antibody 4C1.

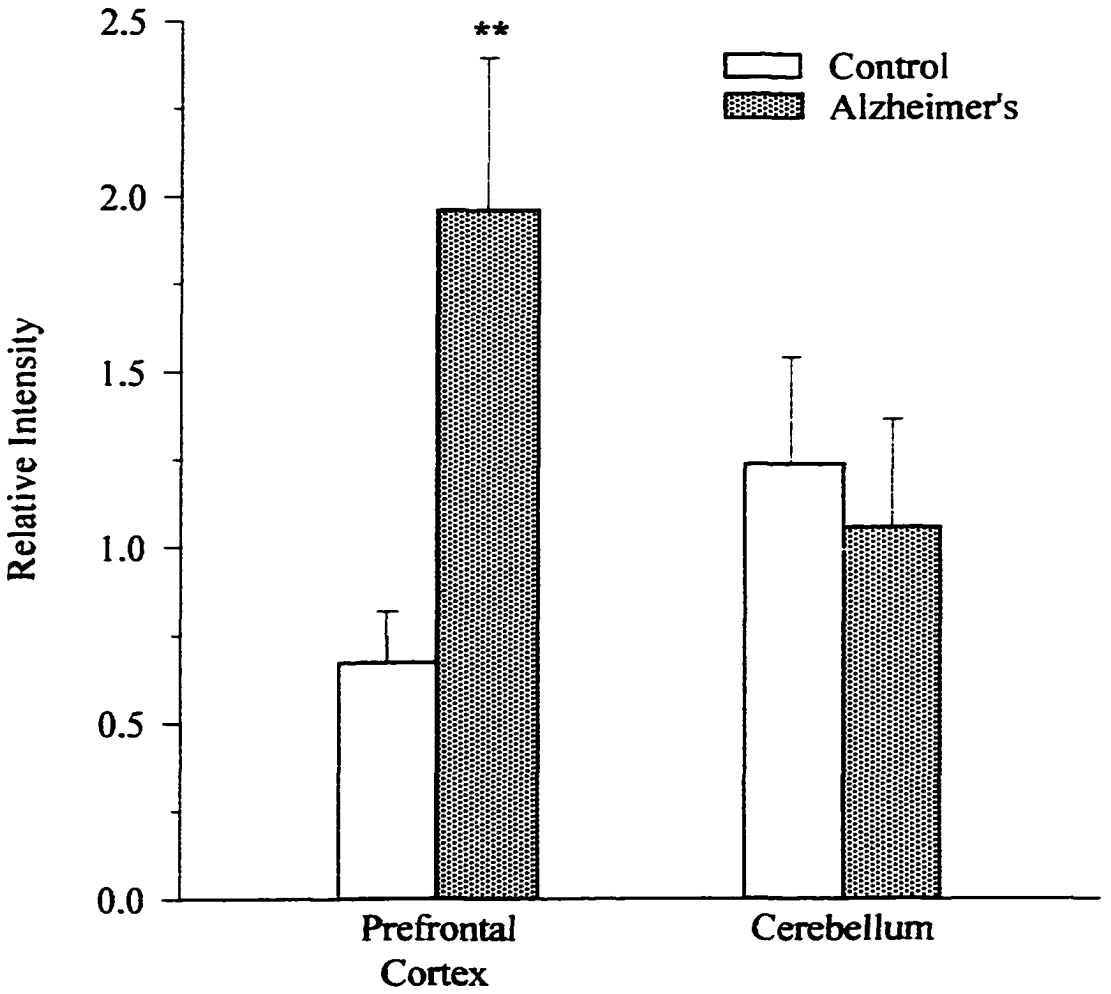

FIG. 4. Quantitative immunoblot analysis of the levels of tissue transglutaminase in the prefrontal cortex and cerebellum of control and Alzheimer's disease cases matched for age and PMI. Tissue transglutaminase levels in the prefrontal cortex were elevated almost three-fold in Alzheimer's disease samples compared to controls. Mean ± SEM. $^{**}p < 0.05$.

DISCUSSION

One of the prevailing unsolved problems in Alzheimer's disease research is the identification of the mechanisms involved in neurofibrillary tangle formation. This is of importance because once it is known how neurofibrillary tangles form, investigators can address the question of why this occurs in Alzheimer's disease. Probably the most widely held view is that posttranslational modifications of τ increases its self-association which initiates the process leading to the formation of PHFs and neurofibrillary tangles (Crowther et al., 1992; Goedert et al., 1992; Goedert, 1993; Schweers et al., 1995). We recently suggested that an enzyme-driven mechanism should be reconsidered as a possible factor in this process because transglutaminase rapidly cross-links τ (Dudek and Johnson, 1993; Miller and Johnson, 1995). Furthermore, τ modified by transglutaminase formed insoluble, filamentous and τ cross-linked with transglutaminase showed enhanced reactivity with Alz-50 (Dudek and Johnson, 1993), an antibody that intensely stains neurons in Alzheimer's, but not in control brains (Wolozin et al., 1986; Love et al., 1988). The latter thus showed that τ cross-linked by transglutaminase takes on a conformation that has been selectively associated with Alzheimer's disease neuropathology. To further examine whether transglutaminase may be involved in the neurodegeneration of Alzheimer's disease, transglutaminase activity and tissue transglutaminase levels were measured in Alzheimer's disease and matched control cortices, where neurofibrillary pathology is prevalent (Braak and Braak, 1991), and cerebellum where few if any neurofibrillary tangles are present (Dustin et al., 1992). The results showed that transglutaminase was elevated significantly in Alzheimer's disease brain compared with controls and that this elevation was selectively localized, as it occurred in neurofibrillary tangle-bearing cerebral cortex, but not neurofibrillary tangle-free cerebellum.

A variety of evidence indicates that transglutaminase is a normal constituent of neurons and has been implicated as functioning in a number of different functional roles. Transglutaminase activity is present both in the central and peripheral nervous system

(Selkoe et al., 1982*a*; Gilad and Varon, 1985; Gilad et al., 1985; Ohashi et al., 1995) and is developmentally regulated (Gilad and Varon, 1985; Gilad et al., 1985). Transglutaminase activity is enriched in synaptosomal preparations compared to other subcellular fractions (Gilad and Varon, 1985), and in cultured cerebellar granule neurons, tissue transglutaminase has been immunolocalized to axons (Perry et al., 1995). Neuronal transglutaminase has been proposed to modulate synaptic plasticity and neurotransmitter release (Pastuszko et al., 1986; Facchiano et al., 1993), contribute to long-term potentiation (Friedrich et al., 1991), and play a role in axonal regeneration (Gilad et al., 1985; Ando et al., 1993). These data clearly indicate that transglutaminase most likely is involved in the normal functioning of neurons.

There are three compelling properties of transglutaminase which support the possibility of its playing a role in Alzheimer's disease neurodegeneration: it is activated by calcium, its activity is increased during apoptosis, and oxidation of proteins increases their susceptibility to cross-linking by transglutaminase. Tissue transglutaminase is a calcium-dependent enzyme (Folk and Finlayson, 1977), and there is a great deal of evidence indicating that calcium homeostasis is disrupted in Alzheimer's disease, causing increased intracellular levels of calcium (for a review, see Mattson, 1992). Because transglutaminase is calcium-activated and because τ is an excellent substrate of this enzyme in vitro (Miller and Johnson, 1995), it can be hypothesized that the increased intracellular calcium levels in Alzheimer's disease activates transglutaminase which may be involved in the conversion of τ from its normal, soluble form to a polymerized, insoluble form, such as that found in neurofibrillary tangles. Interestingly, as stated above, Alz-50 immunoreactivity, is significantly increased when τ is cross-linked by transglutaminase in vitro (Dudek and Johnson, 1993). In addition, transglutaminase activity has been shown to increase significantly during apoptosis (Fesus et al., 1987; El Alaoui et al., 1992). The correlation between increased transglutaminase activity and apoptosis is intriguing for several reasons. First, β-amyloid peptides (A β), which are deposited extracellularly in Alzheimer's brain to form

abundant senile plaques (Glenner and Wong, 1984), disrupt calcium homeostasis in cultured neurons (Mattson et al., 1992; Mattson, 1994), and induce apoptosis (Loo et al., 1993). Second, the mechanism by which A β impairs calcium homeostasis and induces cell death most likely involves increased production of reactive oxygen species (ROS) (Goodman and Mattson, 1994; Mark et al., 1995). Markers of protein oxidation have been shown to be increased in Alzheimer's cerebral cortex and hippocampus, but not cerebellum, relative to controls (Hensley et al., 1995). Heightened oxidative conditions enhance τ self-association (Guttmann et al., 1995; Schweers et al., 1995), and oxidation of proteins can increase their cross-linking by transglutaminase (Groenen et al., 1993). Additionally, there is evidence to suggest that apoptosis contributes to the neuronal degeneration in Alzheimer's disease (Cotman and Anderson, 1995; Anderson et al., 1996). Taken together these data support the hypothesis that in Alzheimer's disease, increases in intracellular calcium, apoptosis, and protein oxidation could individually or in combination lead to increased transglutaminase activity, and this could contribute to the cross-linking of τ and the formation of the insoluble neurofibrillary tangles.

Tissue transglutaminase is a novel protein in that it exhibits two distinct enzyme activities. In its classical role, tissue transglutaminase can catalyze the cross-linking of substrate proteins or the incorporation of polyamines into a substrate (Greenberg et al., 1991). This activity of tissue transglutaminase is potently inhibited by GTP (Achyuthan and Greenberg, 1987; Lee et al., 1989). In accordance with these reports, transglutaminase activity in human brain was found to be almost completely inhibited by 100 mM GTPγS (data not shown). More recently tissue transglutaminase has been shown to be identical with Gh, a G protein that mediates receptor-coupled signal transduction (Nakaoka et al., 1994), a function exhibited by no other known transglutaminase (Greenberg et al., 1991). Tissue transglutaminase (Gh) has been shown to interact with α1-adrenoreceptors (Nakaoka et al., 1994) and in the presence of GTPγS selectively activates phospholipase C-δ-1 (Feng et al., 1996). Therefore when GTP is bound to tissue

transglutaminase it apparently functions as a G-protein rather than as a cross-linking enzyme. Moreover, the specific activity of phospholipase C-δ is significantly decreased in Alzheimer's disease brain (Shimohama et al., 1995), and phospholipase C-δ has been immunolocalized to the neurofibrillary tangles, the neurites surrounding senile plaques and neuropil threads (Shimohama et al., 1991). Thus, it is intriguing that both τ, a substrate for transglutaminase, and phospholipase C-δ, an effector enzyme of Gh (tissue transglutaminase), show some co-localization in the neuropathological structures of Alzheimer's disease. Considering these findings, it is tantalizing to speculate that in Alzheimer's disease the interconversion between tissue transglutaminase and Gh is shifted towards transglutamination so phospholipase C-δ activity is lower than in controls. In addition, it can be suggested that the enzyme's transglutamination function may be enhanced in Alzheimer's disease, contributing to the cross-linking of τ and formation of the insoluble neurofibrillary tangles and at the same time localizing phospholipase C-δ to these structures.

In summary, we have demonstrated for the first time that in the prefrontal cortex of Alzheimer's disease brain transglutaminase activity and the levels of tissue transglutaminase are significantly elevated. Finally, the novel role of tissue transglutaminase in phospholipase C-δ activation (Feng et al., 1996) also needs to be investigated as a possible mechanism by which signal transduction may be disrupted in Alzheimer's disease (Jope, 1996).

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A RAPID, SINGLE-STEP PROCEDURE FOR THE IDENTIFICATION OF TRANSGLUTAMINASE-MEDIATED ISODIPEPTIDE CROSS-LINKS IN AMINO ACID DIGESTS

by

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In preparation for *Journal of Chromatography B*

Format adapted for dissertation

ABSTRACT

Tissue transglutaminase is a calcium-activated, thiol dependent enzyme which can covalently cross-link the ε-amino group of a peptide-bound lysine into the γ-carboxamide group of a peptide-bound glutamine. The resulting ε-(γ-glutamyl)lysine isopeptide bonds confer the properties of insolubility and protease resistance to tissue transglutaminase substrates. A key step in determining whether a putative substrate is actually cross-linked by tissue transglutaminase is the detection of the ε-(γ-glutamyl)lysine isodipeptide in enzymatically proteolyzed protein digests. We have developed a rapid and sensitive method for the isolation and detection of tissue transglutaminase-mediated isodipeptide bonds from purified proteins and tissue homogenates. By using enzymatic proteolysis, derivatization with phenylisothiocyanate, and a single-step elution gradient for high-performance liquid chromatography (HPLC), the frequency of cross-links in purified fibrin was determined to be similar to that reported using a more traditional approach. This method was also used to determine the frequency of cross-linking in purified τ proteins incubated with tissue transglutaminase and to isolate isodipeptide bonds from whole-tissue liver homogenates.

INTRODUCTION

The transglutaminases are a family of calcium-activated, thiol dependent enzymes which can cross-link substrate proteins into high molecular weight complexes that are insoluble and protease-resistant (Folk and Finlayson, 1977). Six distinct transglutaminase genes have been identified in mammals, including epidermal, keratinocyte, prostate, and tissue type transglutaminase, blood coagulation factor XIIIa (Fesus et al., 1996), and a newly-discovered transglutaminase X, which was found in human keratinocytes using PCR (Aeschlimann et al., 1998). Various transglutaminases are involved in skin, hair, and nail cornification; hepatic fibrogenesis; fibrin cross-linking during clot formation; and the

formation of cataracts in the eye lens (for a review, see Greenberg et al., 1991; Fesus et al., 1996; Melino and Piacentini, 1998).

The binding of calcium by transglutaminase exposes an active-site cysteine residue which can react with the γ-carboxamide of a substrate glutamine, forming an intermediate γ-glutamyl thioester and releasing ammonia (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Greenberg et al., 1991). The acyl-enzyme intermediate then reacts with a nucleophilic primary amine, resulting in an incorporation of the amine-containing "donor" into the substrate glutamyl "acceptor." If the primary amine is donated by the ε-amino group of a peptide-bound lysine, the result is a cross-linking of the two proteins via the formation of a ε-(γ-glutamyl)lysine isopeptide bond. Because this isopeptide bond is a unique product of transglutaminase activity, the detection of such bonds is a key step in determining whether a putative substrate can be cross-linked by the enzyme.

As the transglutaminase-mediated cross-linking of proteins produces a covalent modification between the side chains of glutamine and lysine, the resulting ε-(γ-glutamyl)lysine isopeptide bonds are not susceptible to cleavage by proteases which hydrolyze ordinary peptide bonds. Thus, isolation and detection of ε-(γ-glutamyl)lysine is generally accomplished using exhaustive proteolysis of the cross-linked proteins, followed by chromatographic purification of the isodipeptide (Loewy, 1984). Several chromatographic methods have been used to isolate the isodipeptide, including paper, thin-layer, and ion-exchange chromatography (Loewy, 1984). However, as the transglutaminase-mediated cross-link generally occurs in a low stoichiometry, the most reliable method of isolation involves reverse-phase high-performance liquid chromatography (RP-HPLC) (Loewy, 1984; Tarcsa and Fesus, 1990).

Because the single amino acids generated by exhaustive proteolysis cannot be easily detected using a spectrophotometer, derivitization of the amino acids prior to detection is necessary. Precolumn derivitization with *o*-phthalaldehyde (OPA) has proved to be useful for such detection (Griffin et al., 1982; Beninati et al., 1988); however, this protocol

requires the presence of a fluorescence monitor, and OPA does not react with proline and hydroxyproline residues (Bidlingmeyer et al., 1984). Furthermore, the fluorescent products are relatively unstable and present difficulties in quantification due to the sensitivity to quenchers (Heinrikson and Meredith, 1984). To overcome these problems, an alternate derivitization technique using phenylisothiocyanate (PITC) has also been developed (Bidlingmeyer et al., 1984; Heinrikson and Meredith, 1984). This method allows for rapid and reproducible detection of low levels of amino acids, and the phenylthiocarbamyl (PTC) derivatives are relatively stable at 4°C (Cohen et al., 1986).

Derivatization of amino acids with PITC has been successfully employed in the detection of ε-(γ-glutamyl)lysine bonds (Tarcsa and Fesus, 1990). Unfortunately, the elution conditions used in these studies do not achieve optimal separation of derivatized enzyme-digested amino acids in the vicinity of the isodipeptide. The ε-(γ-glutamyl)lysine isodipeptide elutes as a very weak shoulder on a large neighboring amino acid peak (Tarcsa and Fesus, 1990; Tarcsa et al., 1992). The method also requires a preliminary purification step using a cation exchanger resin, as well as HPLC separation on a silica column prior to derivatization.

In the present study, PITC-derivatized amino acid samples are separated using RP-HPLC, and a simple one-step elution gradient is employed to achieve excellent separation of the ε-(γ-glutamyl)lysine isodipeptide from adjacent amino acids. No prior purification steps are necessary, and the entire process is completed in 60 min, including wash steps before and after the elution. Using this new method, the ε-(γ-glutamyl)lysine isodipeptide was isolated from purified human fibrin, and the extent of cross-linking (1.7 moles of isodipeptide per mole of fibrin) is similar to that determined previously (Lorand et al., 1968). The extent of isodipeptide formation was also determined after the in vitro cross-linking of the τ protein by tissue transglutaminase. τ, a microtubule-associated protein, has been shown to be a substrate of tissue transglutaminase, a reaction which may be of potential relevance to Alzheimer's disease (Miller and Johnson, 1995).

Approximately 0.75 moles of isodipeptide is incorporated per mole of τ. Isodipeptide bonds were also isolated from the crude liver homogenate of a 2-year old rat, indicating that the present method is useful in detecting the presence of isodipeptide in physiological samples as well.

EXPERIMENTAL

Materials

Guinea pig liver tissue transglutaminase, fibrin (human plasma, washed), bovine serum albumin (BSA), PITC, triethylamine (TEA), sodium acetate, methanol, acetonitrile, and individual amino acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amino acid standard-H and the BCA Protein Determination Kit were purchased from Pierce (Rockford, IL, USA). The isodipeptide ε-(γ-glutamyl)lysine, as well as glutamine-ε-lysine and glu-lys dipeptide, were purchased from Bachem Bioscience (King of Prussia, PA, USA). All proteases were purchased from Boehringer Mannheim (Indianapolis, IN, USA) except leucine aminopeptidase (Sigma-Aldrich) and carboxypeptidase A (Worthington Biochemical Corp., Lakewood, NJ, USA). τ from bovine brains was purified as described previously (Johnson et al., 1989), and the recombinant human τ isoform T4L (Goedert et al., 1989) was expressed and cloned as described by Fleming et al. (1996). Liver from a 2-year old Sprague-Dawley rat which had been used for hepatotoxin studies was kindly donated by Dr. Michael Wyss of the University of Alabama at Birmingham.

Equipment

Peptide digests and amino acid standards were dried and derivatized using the Waters (Milford, MA, USA) Pico-Tag System. Separation was achieved using a DX300 HPLC system, with a GPM gradient pump module and a VDM-II variable wavelength detector (Dionex Corp., Sunnyvale, CA, USA). The Vydac (Hesperia, CA, USA) reverse

phase C-18 column (218TP-5405, 5 micron, 4.6 x 50 mm) was maintained at 30°C using a column heater (Dionex). Derivatized PTC-amino acids were detected at 254 nm.

Chromatographic conditions

Amino acid digests were separated using the program outlined in Table I. Buffer 1 consisted of 70 mM ammonium acetate (pH 6.55), 2.5% acetonitrile, 0.2 µg/mL EDTA, and 0.05% triethylamine. Buffer 2 was 45% acetonitrile, 15% methanol in water. Buffers were degassed by sonication under vacuum for 1 min.

TABLE 1

Elution conditions

Time	Flow rate	% Buffer 1	% Buffer 2	Comments
0.0	0.1	0	100	Wash
0.1	1.0	0	100	
2.5	1.0	0	100	
2.6	1.0	100	0	Equilibrate
10.0	1.0	100	0	Reset Absorbance
10.1	1.0	100	0	Inject
19.0	1.0	78	22	
34.0	1.0	68	32	
41.0	1.0	56	44	
42.5	1.0	0	100	Wash
50.0	1.4	0	100	
54.0	1.4	0	100	
60.0	1.4	0	100	

Standard solutions and calibration

Using the method of Heinrikson and Merdith (1984), amino acid preparations were dried under vacuum and redissolved in 10-20 µl of a drying solution containing 1 M

sodium acetate, methanol, and triethylamine in a ratio of 2:2:1. The samples were again dried under vacuum, then reconstituted in a freshly-made derivatizing solution containing methanol, water, triethylamine, and PITC in a ratio of 7:1:1:1. Derivatization was performed at room temperature for 10 min, using occasional sonication and repeated vortexing to ensure complete resolubilization. The samples were dried as above, then redissolved in 200 µl of 7:2 water: acetonitrile. Aliquots (usually 5-20 µl) were injected into a reverse phase C-18 column and separated using the program described in Table I. Because of minor batch-to-batch eluent variations and changing elution properties due to settling of the eluents, slight variations in retention times (less than ± 0.5 min) were occasionally noticed. In all experiments, amino acid standards and purified isodipeptide were used to calibrate the elution profile prior to data analysis.

Sample preparation

Bovine τ and the recombinant human τ isoform T4L were cross-linked by tissue transglutaminase as previously described (Miller and Johnson, 1995). Cross-linked bovine τ (5 mg/ml), fibrin (10 mg/ml), BSA (10 mg/ml), or cross-linked T4L (1 mg/ml) were subjected to exhaustive enzymatic proteolysis. Several different combinations of proteases have been successfully used by this laboratory, and the results are consistent, provided that complete proteolysis into amino acids occurs.

The protocol for proteolyzing cross-linked bovine τ was as follows: Bovine τ (1 mg) which had been cross-linked by tissue transglutaminase was incubated in 200 µl of a buffer containing 10 mM Tris (pH 7.0) and a small crystal of thymol to inhibit bacterial growth. Pronase was added (1:20), and the solution was incubated at 37°C for 24 h. The pH of the buffer was adjusted to 8.0, and proteinase K was added (1:20) and incubated for 24 h at 50°C. Magnesium chloride was added to a concentration of 5 mM, and the sample was incubated with leucine aminopeptidase (1:25) for 24 h at 37°C. Carboxypeptidases A and Y were added (1:25) and incubated for 1 h at 37°C. As a control for proteolysis of the

proteases themselves, a parallel sample was prepared which contained the proteases alone. Between each addition of protease, the samples were boiled for 5 min to inactivate the previous protease, then sonicated. The final samples were diluted to the same volume to compensate for evaporation, and the samples were centrifuged through 10 kDa filters (Waters Corporation). An aliquot of each sample (30 µl) was combined with 2 µl of 500 mM NaCl (to prevent freezing during derivatization) and 30 nmol of norleucine, an internal standard. The samples were derivatized and isodipeptide content was determined as described above.

Preparation of physiological tissue samples

As apoptotic hepatocytes are known to contain substantial amounts of tissue transglutaminase-mediated ε-(γ-glutamyl)lysine isodipeptide bonds (Fesus et al., 1989), the liver of a 2-year old Sprague-Dawley rat which had been used for hepatotoxin studies was utilized as a physiological tissue sample in our experiments. The liver was removed, weighed, and homogenized as described by Fesus and Arato (1986). As a positive control, a portion of the homogenate (0.325 g) was incubated with tissue transglutaminase (1:1000) in the presence of 2 mM exogenous $CaCl_2$ for 10 min at 37°C. Tween-20 was added to 0.1%, and the samples were sonicated. Protein concentrations were determined using the BCA method (Pierce). Total protein (6.0 mg each) was precipitated at 4°C for 30 min using 10% trichloroacetic acid (TCA) in a volume of 400 µl. The pellets were washed three times with 600 µl of diethyl ether and dried overnight in a fume hood. As a further positive control, purified isodipeptide (2 nmol / mg protein) was exogenously added to one of the samples which had not been incubated with tissue transglutaminase. Each of the samples was exhaustively proteolyzed in 200 µl of a buffer containing 10 mM Tris (pH 8.0), 0.1% SDS, and a small crystal of thymol. These samples, as well as a blank sample to control for the proteolysis of the proteases themselves, were incubated at 37°C (unless otherwise indicated) with the following proteases: pronase (1:50, 24 h), proteinase K (1:50, 24 h,

50°C), subtilisin (1:100, 24 h), proteinase K again (1:100, 24 h, 50°C), leucine aminopeptidase (1:200, 24 h, with 5 mM $MgCl_2$), carboxypeptidase Y (1:100, 2.5 h), carboxypeptidase A (1:100, 1.5 h), and carboxypeptidase B (1:500, 6.5 h). Samples were concentrated using a speed-vac centrifuge, rediluted to 200 µl, and processed as above, except that the derivatization time was extended to 20 min.

Acid hydrolysis

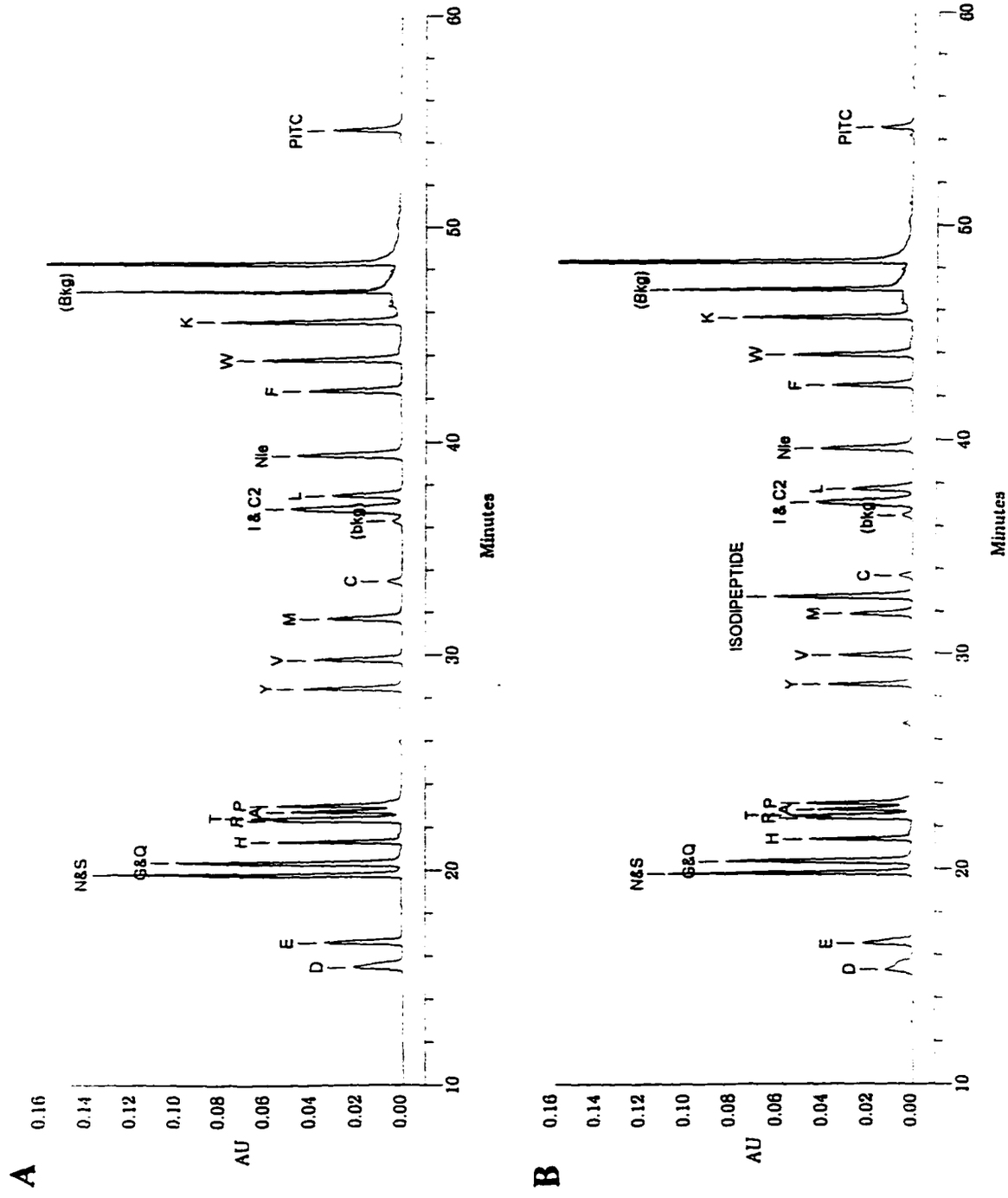
Acid hydrolysis of amino acid digests was performed by incubating the samples in the presence or absence of 6 N hydrochloric acid (HCl), at 110°C for 18 h. The samples were blanketed with nitrogen and sealed in glass conical vials prior to hydrolysis.

RESULTS AND DISCUSSION

Mixtures containing the acid-hydrolyzed amino acid standards were supplemented with asparagine, cysteine, glutamine, tryptophan, and norleucine internal standard, with and without the addition of ε-(γ-glutamyl)lysine isodipeptide. These samples were derivatized with PITC and separated using the HPLC elution program indicated in Table 1. A typical chromatographic pattern of these samples is shown in Fig. 1. The retention times of the amino acids were verified by derivatizing and applying them individually to the HPLC. The isodipeptide elutes as a sharp, well-resolved peak between methionine and cysteine. As cysteine is generally converted to cystine during PITC derivatization, the peak due to this amino acid is minimal. Under the chromatographic conditions used, the isodipeptide is clearly separated from other similar molecules, such as glutamine-ε-lysine and glu-lys dipeptide (data not shown). The concentration curve of isodipeptide is shown in Fig. 2. The curve is linear between 15-250 pmol of isodipeptide, and the limit of detection for purified isodipeptide is between 10 and 15 pmol.

To verify that the HPLC method is useful for the isolation of physiological isodipeptide, washed human fibrin, which is known to contain ε-(γ-glutamyl)lysine isopeptide

FIG. 1. Typical chromatographic pattern of PITC-derivatized amino acid standards, in the absence **(A)** or presence **(B)** of purified PITC-derivatized ε-(γ-glutamyl)lysine isodipeptide. The isodipeptide peak is well-resolved and elutes between methionine and cystine.



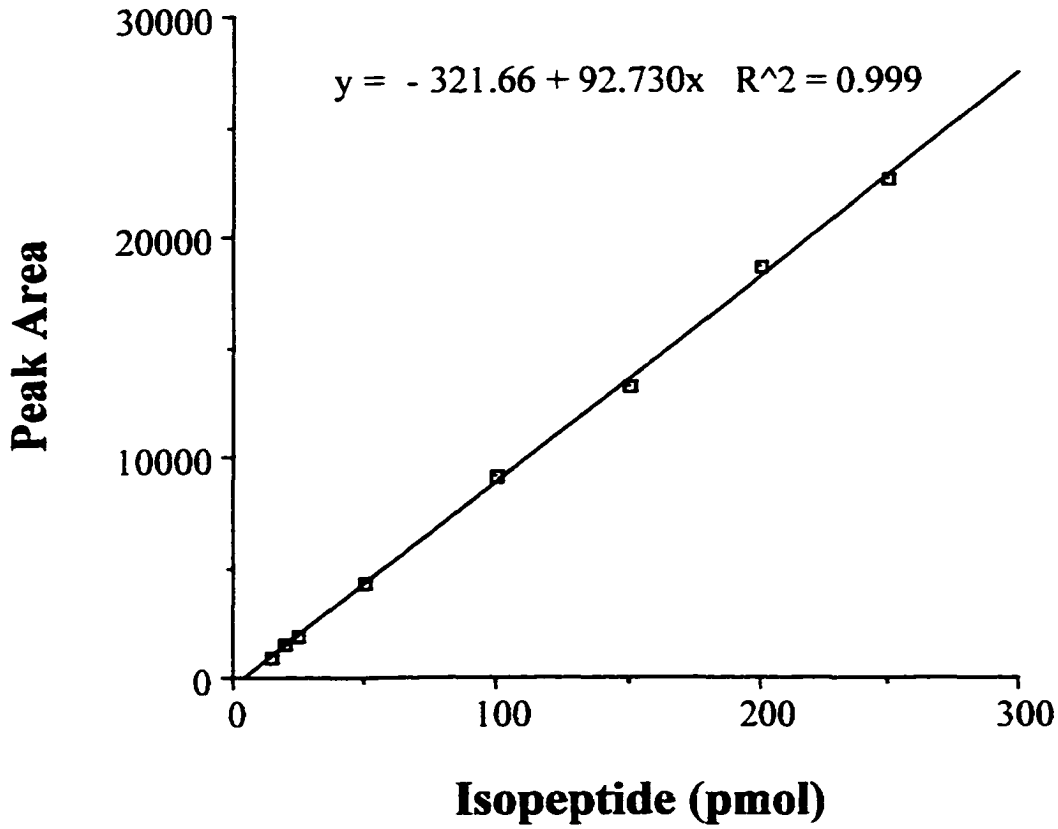
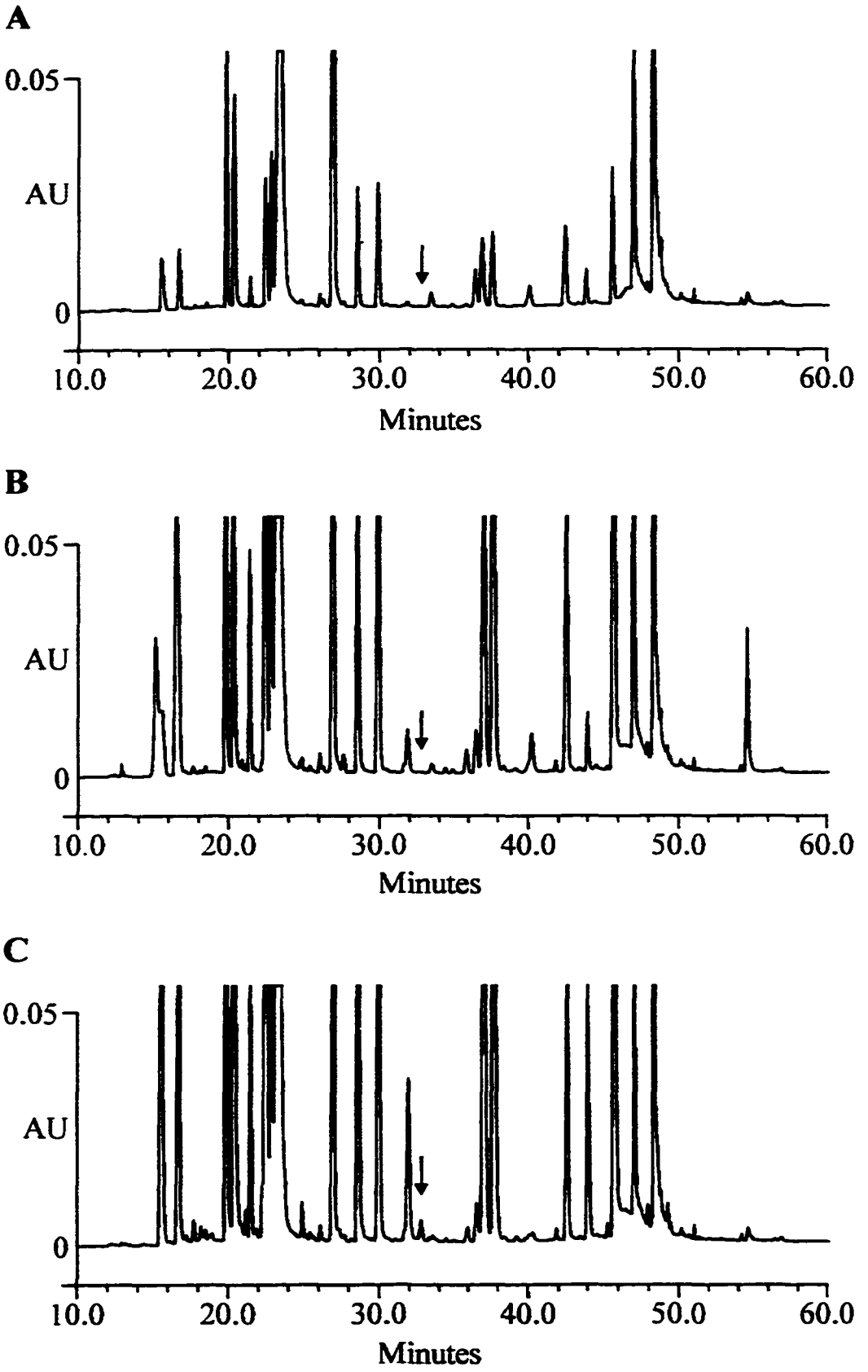


FIG. 2. Calibration curve for the quantification of purified PITC-derivatized ε-(γ-glutamyl)lysine. The curve is linear in the range of 15-250 pmol.

bonds (Lorand et al., 1968), and BSA, which does not contain isodipeptide bonds (Miller and Johnson, 1995), were enzymatically proteolyzed into amino acids. After derivatization and HPLC separation, the amino acid digest of fibrin was found to contain a significant concentration of isodipeptide, whereas the digests of BSA and the proteases alone did not (Fig. 3). Based upon the calibration curve shown in Fig. 2, 1 nmol of fibrin was determined to contain 1.7 ± 0.3 nmol of isodipeptide. This result is comparable to a previously published result of 1-1.4 nmol of isodipeptide per nmol of fibrin (Lorand et al., 1968).

The microtubule-associated protein τ has also been shown to be a substrate of tissue transglutaminase, and it has been proposed that tissue transglutaminase cross-linking may be involved in the formation of insoluble neurofibrillary tangles in Alzheimer's disease (Selkoe et al., 1982; Miller and Johnson, 1995). To determine the stoichiometry of isodipeptide formation by tissue transglutaminase in τ, soluble bovine τ or human recombinant τ isoform T4L (Goedert et al., 1989) were incubated in vitro with or without calcium and tissue transglutaminase, and the resulting proteins were subjected to proteolysis and analyzed. Cross-linked bovine τ (Fig. 4, upper trace) and the cross-linked human τ isoform (data not shown) were found to contain comparable amounts of the isodipeptide, whereas no isodipeptide was detected in the τ sample which had not been incubated with tissue transglutaminase (Fig. 4, lower trace). The frequency of isodipeptide formation in cross-linked τ was determined to be 0.75 ± 0.12 mol of isodipeptide per mole of τ in our experiments. This value is significantly lower than the number of glutamines within τ that can be modified by tissue transglutaminase, as determined by polyamine incorporation assays (Miller and Johnson, 1995; Murthy et al., 1998). Considering that cross-linking is a highly specific occurrence, not all potential substrate glutamines and lysines would be expected to participate. As polypeptide regions of substrate proteins associate, only substrate glutamines which are closely apposed to substrate lysines will become cross-linked. The data from these experiments indicate that τ is cross-linked at a

FIG. 3. Chromatographic patterns of the PITC-derivatized enzymatic digests of BSA (B) and fibrin (C) and of the proteases alone (A). Aliquots corresponding to approximately 17 μg of total protein were injected onto the HPLC. A peak corresponding to derivatized ε-(γ-glutamyl)lysine isodipeptide can be seen in the elution profile of fibrin, but not in the profile of BSA or the protease control (arrows).



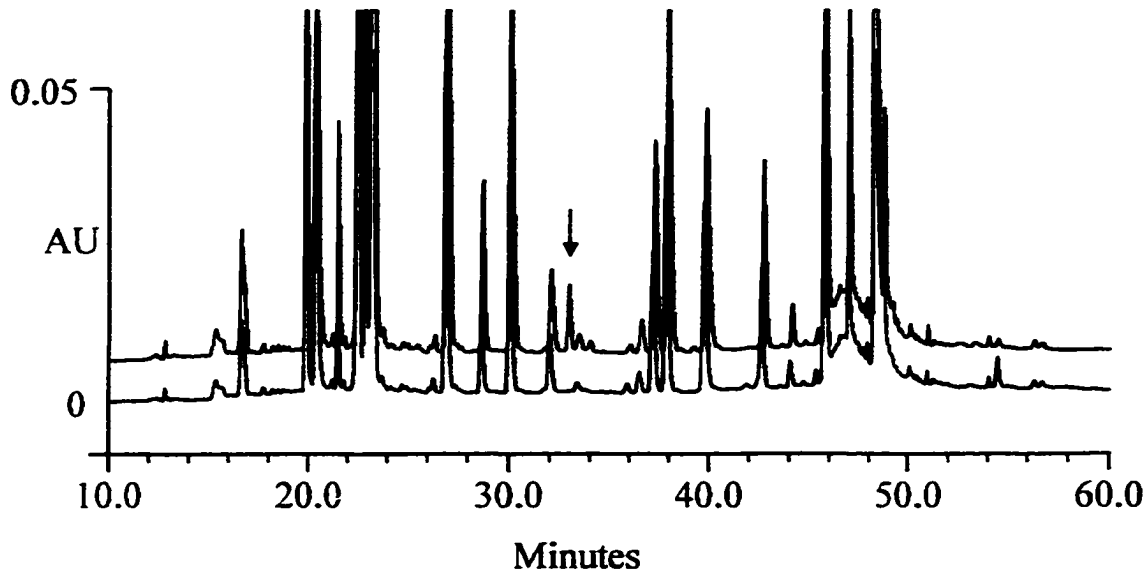


FIG. 4. Chromatographic patterns of the PITC-derivatized enzymatic digests of tissue transglutaminase-cross-linked (upper trace) and non-cross-linked (lower trace) bovine τ. Aliquots corresponding to approximately 7 μg of total protein were injected onto the HPLC. The ε-(γ-glutamyl)lysine isopeptide bond (arrow) is present in cross-linked bovine τ, but not in τ which has not been cross-linked.

sub-stoichiometric ratio, and it is clear that not every site within τ that can be modified by tissue transglutaminase is actually cross-linked by the enzyme. It is also likely that the kinetics of τ association and other reaction conditions may have inhibited complete cross-linking by tissue transglutaminase.

A necessary requirement for isodipeptide isolation is the ability to identify them in physiological tissue samples. Because apoptotic hepatocytes are known to contain substantial amounts of ε-(γ-glutamyl)lysine isodipeptide bonds (Fesus et al., 1989), the liver of a 2-year-old Sprague-Dawley rat which had been used for hepatotoxin studies was utilized as a physiological tissue sample in our experiments. As a positive control, a portion of the liver homogenate (0.325 g) was incubated in the presence of calcium and tissue transglutaminase, to further enhance the amount of isodipeptide in the sample. Total protein (6 mg) was acid-precipitated from each homogenate, and, as a further positive control, purified isodipeptide was exogenously added to one of the samples which had not been incubated with tissue transglutaminase. The samples were then subjected to exhaustive enzymatic proteolysis, derivatized, and analyzed.

As seen in Fig. 5A, a significant amount of isodipeptide can be detected in the homogenate of aged rat liver. According to the calibration curve shown in Fig. 2, the concentration of isodipeptide is approximately 0.5 nmol of isodipeptide per mg of total protein. The homogenate which had been incubated with tTG (Fig. 5B) was found to contain 66% more isodipeptide than the non-cross-linked homogenate, and the homogenate which had been spiked with isodipeptide prior to proteolysis (Fig. 5C) was found to contain over 350% more cross-link.

Because ε-(γ-glutamyl)lysine isodipeptide bonds are acid labile, the amino acid digest from aged rat liver homogenate was incubated in the presence or absence of 6 N HCl, at 110°C for 18 h. Incubation in the absence of HCl did not cause any changes in the chromatographic pattern (Fig. 6A). The treatment of the amino acid digest with HCL (Fig. 6B) caused a disappearance of the isodipeptide peak. Unfortunately, incubation with acid

FIG. 5. Chromatographic patterns of the PITC-derivatized enzymatic digests of a liver homogenate from an aged rat. Aliquots corresponding to approximately 170 μg of total protein were injected onto the HPLC. **A:** A significant amount of ε-(γ-glutamyl)lysine isodipeptide can be detected in the aged rat liver. **B:** Rat liver homogenate was incubated in the presence of tissue transglutaminase and calcium prior to digestion. The amount of isodipeptide detected increased by 66%. **C:** As a control, purified isodipeptide (2 nmol / mg protein) was added to the non-cross-linked liver homogenate prior to digestion. Arrows indicate the position of ε-(γ-glutamyl)lysine.

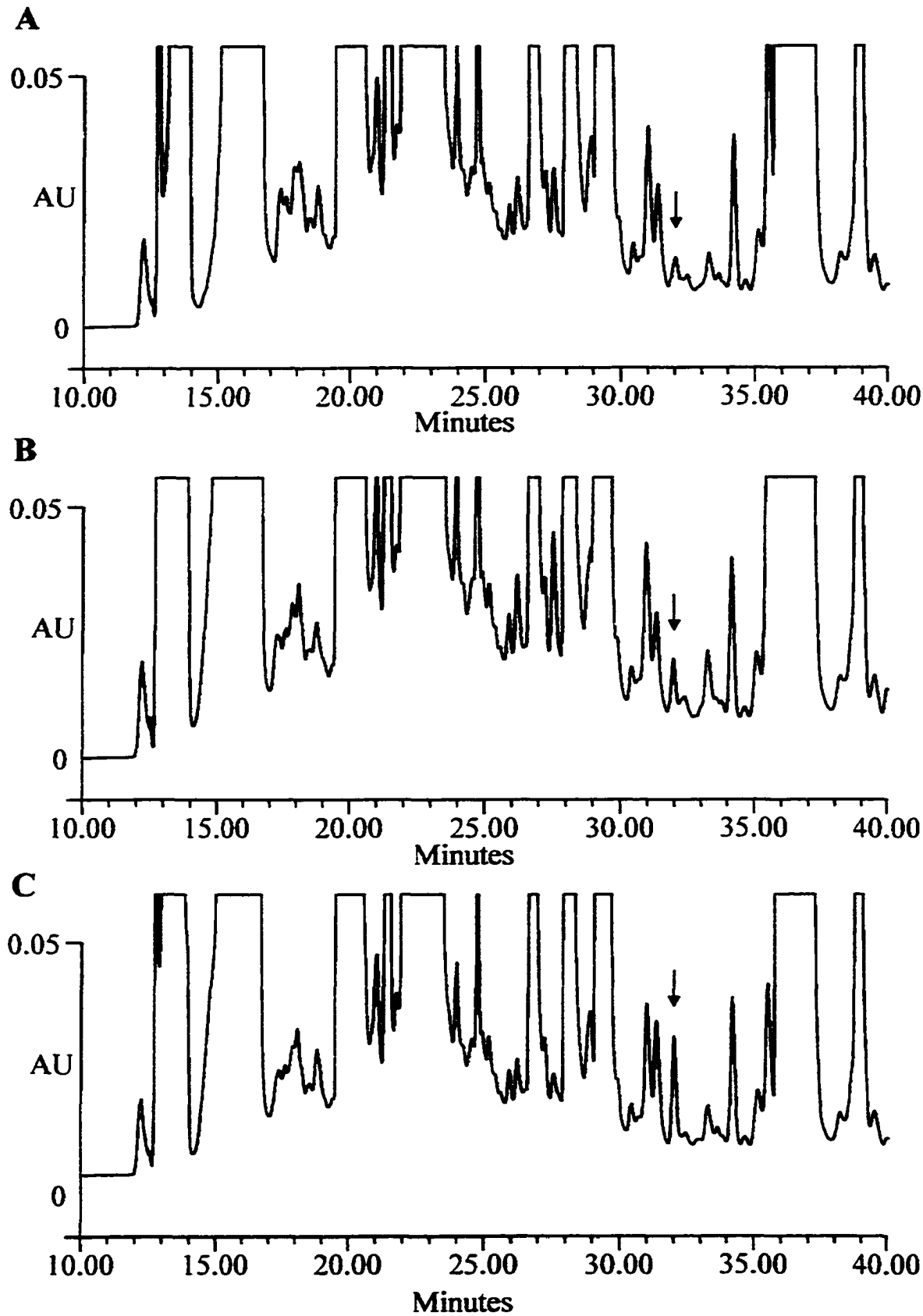
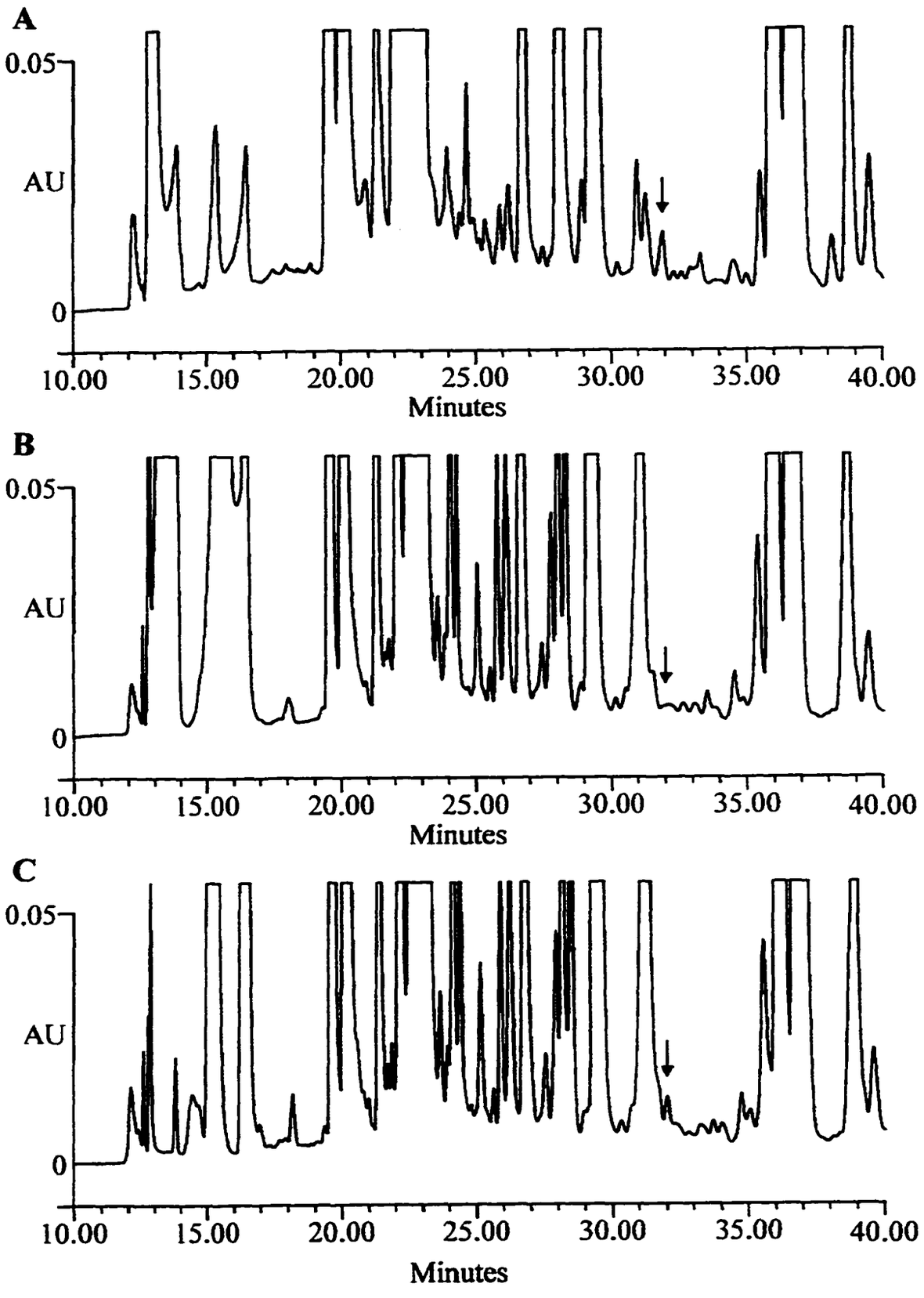


FIG. 6. Chromatographic patterns verify that the ε-(γ-glutamyl)lysine isodipeptide is acid-labile. Prior to derivatization, amino acid digests of rat liver homogenate were acid-hydrolyzed in the absence or presence of 6 N HCl. **A:** Homogenate incubated in the absence of HCl was identical to that seen in Fig. 5A. **B:** The peak corresponding to ε-(γ-glutamyl)lysine is abolished by acid treatment. **C:** Quantitative recovery of the isodipeptide peak by the addition of purified ε-(γ-glutamyl)lysine. Arrows indicate the position of ε-(γ-glutamyl)lysine.



also caused a slight change in the elution of certain peaks, including a large peak which elutes immediately before the isodipeptide. This large peak does not interfere with the detection of isodipeptide, however, as the addition of 75 pmol of isodipeptide prior to injection results in a quantitative recovery of the missing peak (Fig. 6C).

Thus, we have developed a rapid, sensitive technique which is useful for the detection of tissue transglutaminase-catalyzed ε-(γ-glutamyl)lysine isodipeptide bonds from enzyme-digested proteins and tissue homogenates. Because the isodipeptide is clearly separated from other homogenate components, this technique offers significantly improved sensitivity over the established PITC derivatization method (Tarcsa and Fesus, 1990; Tarcsa et al., 1992). The improved resolution obviates the need for prior purification steps and results in a simpler, more rapid assay. Such a method should enable new laboratories to undertake the analysis of isodipeptide bonds without the need for costly fluorescence detectors or multiple column systems.

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CONCLUSION

One of the major neuropathological events in the progression of Alzheimer disease is the assembly of soluble τ into insoluble, protease-resistant PHFs. The purpose of the present group of studies was to establish a model system for the conversion of τ into stable, insoluble polymers. Tissue transglutaminase is a cross-linking enzyme which can covalently incorporate the ε-amino group of a peptide bound lysine into the γ-carboxamide group of a substrate glutamine, forming an ε-(γ-glutamyl)lysine isopeptide bond. Such bonds often confer the properties of stability, insolubility, and protease-resistance to their substrates. For this reason, tissue transglutaminase was investigated as an enzyme which may potentially be involved in Alzheimer disease.

We have determined that bovine τ and recombinant human τ isoforms are excellent substrates of tissue transglutaminase in vitro, with bovine τ demonstrating a *K*m for polyamine incorporation of 10.4 ± 2.2 μM and a Vmax of 40.9 ± 4.5 nmol/mg enzyme/min. Bovine τ and recombinant human τ isoforms rapidly form high molecular weight, cross-linked polymers upon incubation with tissue transglutaminase, and cross-linked τ has an appearance similar to τ from Alzheimer disease brains when visualized on a western blot. Preliminary evidence from this lab also indicates that bovine and recombinant human τ may form filamentous structures when incubated with tissue transglutaminase, although no filaments displaying PHF-like characteristics have been detected thus far (Dudek and Johnson, 1993, and M.L.M. unpublished observations). We originally suggested that the modification of τ by tissue transglutaminase involves one or more glutamine residues in the carboxy-terminal domain of τ (Miller and Johnson, 1995), and these studies have subsequently been verified and extended by others (Lorand, 1996; Murthy et al., 1998).

Although it is well-established that tissue transglutaminase can catalyze both the cross-linking of proteins and the incorporation of polyamines into its substrates, a distinction between these two activities is not always made. Polyamine incorporation studies are used to determine the potential sites of tissue transglutaminase modification within a protein, and it is often tempting to extrapolate from such data that the protein can be cross-linked by tissue transglutaminase. However, our studies indicate that T264, a truncated fragment of recombinant human τ, can incorporate polyamines in a tissue transglutaminase-dependent manner, but cannot be cross-linked by the enzyme. Thus, cross-linking and polyamine incorporation are distinct reactions of tissue transglutaminase, and the ability of a substrate to incorporate polyamines does not necessarily imply that cross-linking also occurs. The differences between polyamine incorporation and cross-linking are also made apparent by the fact that isodipeptide concentrations from cross-linked τ (less than 1 mol per mol of τ) are considerably lower than the amount of polyamine that can be incorporated into τ isoforms (1-3 mol of putrescine per mole of τ) (Miller and Johnson, 1995).

It can be postulated that, in order for cross-linking to occur, polypeptide chains must first be arranged in an orientation such that a substrate glutamine is in close proximity to an available lysine. If this is the case, then only substrate proteins which are already "self-associated" in a specific arrangement would have the potential to be cross-linked by transglutaminase. Substrate glutamine residues without nearby lysines may be able to incorporate polyamines, but might not become cross-linked. This is supported by the fact that the τ construct T264 contains one glutamine and two lysine residues which have been verified as substrates of tissue transglutaminase activity (Murthy et al., 1998), yet T264 is not cross-linked. If cross-linking were a random event or based solely upon the accessibility of substrate residues by tissue transglutaminase, cross-linked polymers would likely be the result.

In agreement with this hypothesis, there is evidence to suggest that τ association is a highly-ordered event, resulting in well-defined filaments even in solution (Lichtenberg-Kraag and Mandelkow, 1990; Wille et al., 1992; Crowther et al., 1994; Schweers et al., 1995). Although in vitro reactions of τ with tissue transglutaminase have not yet been able to produce PHF-like structures (Dudek and Johnson, 1993), it is possible that some other co- or post-translational modification of τ is necessary to induce the periodic helicity seen in such filaments. Alternately, interactions with polyanions such as heparin or RNA (Goedert et al., 1996; Kampers et al., 1996; Hasegawa et al., 1997) may orient τ into a PHF-like conformation, which could then be stabilized by tissue transglutaminase. The importance of τ conformation in Alzheimer disease is evidenced by the discovery that certain "Alzheimer disease-specific" antibodies recognize epitopes which contain both the N-terminus and third binding domain of τ, juxtaposed in a specific orientation which cannot be detected in normal brain (Carmel et al., 1996; Jicha et al., 1997*a*;*b*).

To further investigate the role of tissue transglutaminase in Alzheimer disease, we have measured the protein levels and activity of tissue transglutaminase in Alzheimer disease and control brain tissue. Both the levels and the activity of tissue transglutaminase are significantly elevated in the prefrontal cortex of Alzheimer disease brains, as compared to controls. In the cerebellum, which generally shows no pathology in Alzheimer disease, no differences in tissue transglutaminase protein levels or activity can be detected between Alzheimer disease and control brains. The elevation of tissue transglutaminase levels specifically in brain regions affected by Alzheimer disease suggests that the enzyme may play a compensatory or pathogenic role in the progression of the disease.

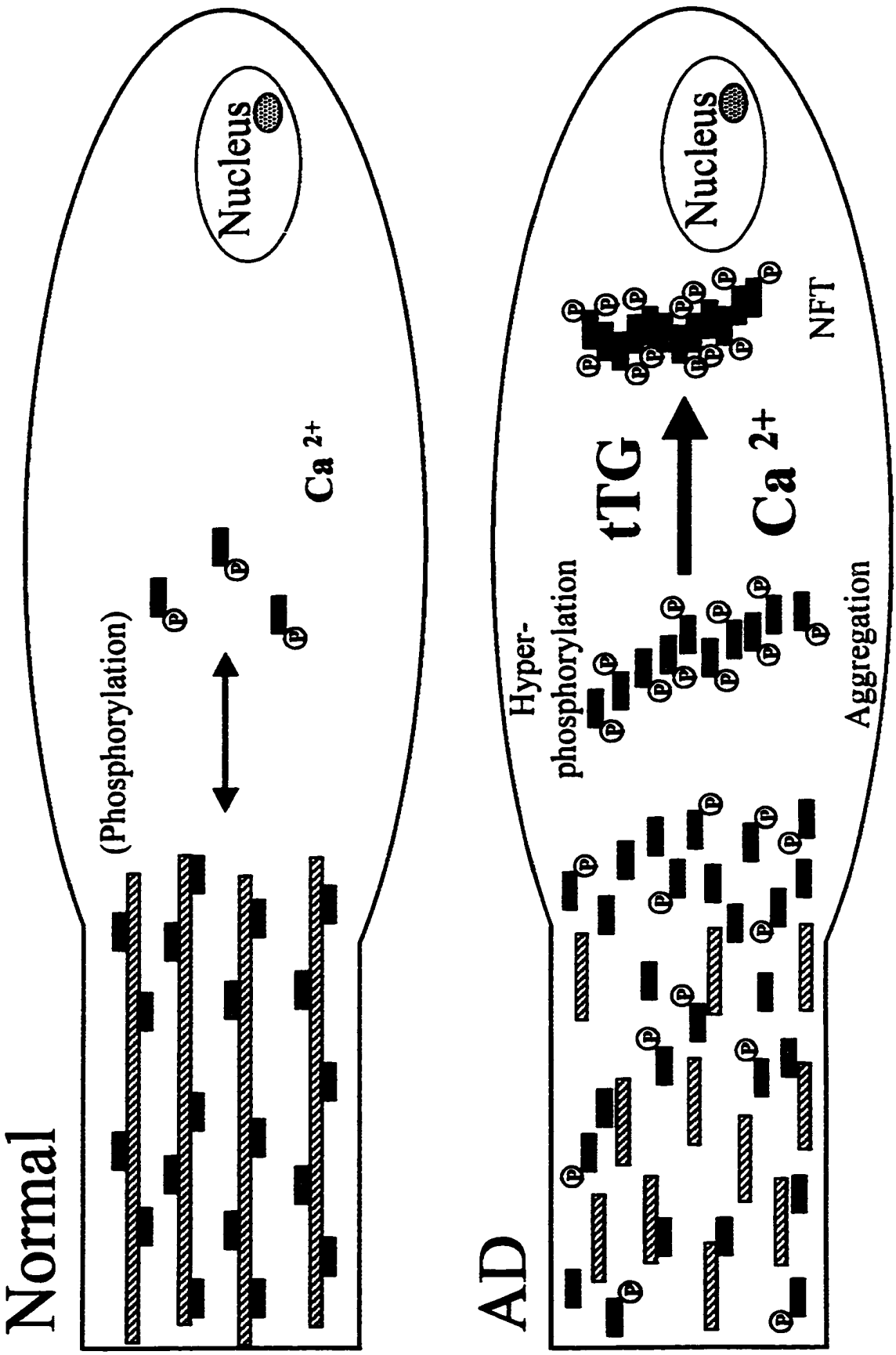
A definitive and necessary step in ascertaining whether tissue transglutaminase is involved in Alzheimer disease pathogenesis is the assay of neurofibrillary tangle preparations for the presence of ε-(γ-glutamyl)lysine isopeptide bonds. We have demonstrated that it is possible to isolate such isodipeptide bonds from purified proteins and tissue homogenates using exhaustive proteolytic digestion, derivatization with phenyliso-

thiocyanate, and separation with high performance liquid chromatography (HPLC). We have also developed an elution gradient for the HPLC which obviates the need for prior amino acid purification steps. Although similar techniques have demonstrated the presence of isodipeptide in fibrin and other cross-linked proteins (Griffin et al., 1982; Beninati et al., 1988; Tarcsa and Fesus, 1990), thus far we have been unable to find an appropriate method for solubilizing and proteolyzing the neurofibrillary tangles. This may be due in part to the extreme insolubility of the NFT-τ (Selkoe et al., 1982*b*), the presence of multiple phosphate groups which may restrict the access of proteases (Hasegawa et al., 1992; Hanger et al., 1998), or the association of neurofibrillary tangles with glycolipids, which are not affected by protease activity (Goux et al., 1996).

The proposed role of tissue transglutaminase in the formation of neurofibrillary tangles is diagrammed in Fig. 1. Cellular changes in Alzheimer disease include the break-down of the microtubule network, hyperphosphorylation of τ, and the somatodendritic accumulation of τ polymers, although the temporal order of these events is not yet clear. According to this model, increased intracellular calcium concentrations cause an abnormal activation of tissue transglutaminase, which may itself be upregulated due to a variety of factors in Alzheimer disease. The active tissue transglutaminase then cross-links soluble PHFs into insoluble, protease-resistant PHFs to form the core of the neurofibrillary tangles.

As evidence for the role of tissue transglutaminase in Alzheimer disease continues to mount, attempts to dissect the neurofibrillary tangle become increasingly important. Whether or not NFT-τ is ultimately found to contain the isodipeptide bond, tissue transglutaminase cross-linking is a useful tool and model system for the investigation of stable τ polymers. Furthermore, the analytical techniques employed in this work provide a protocol which may be useful in dissecting a variety of other paradigms in which tissue transglutaminase plays a putative role.

FIG. 1. Schematic diagram of the putative role of tissue transglutaminase in Alzheimer disease. Cellular changes occurring in Alzheimer disease include the breakdown of microtubules (hatched bars) and the hyperphosphorylation (P) and somatodendritic aggregation of tau (gray boxes). The aggregated, hyperphosphorylated tau can be found in the form of soluble PHFs, which may be precursors of the insoluble neurofibrillary tangles (NFT). According to this hypothesis, increased intracellular calcium concentrations in Alzheimer disease cause an abnormal activation of cytoplasmic tissue transglutaminase (tTG), which then crosslinks the soluble PHFs into condensed neurofibrillary tangles.



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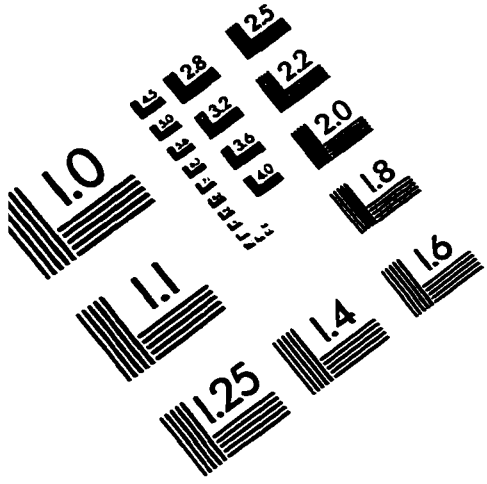

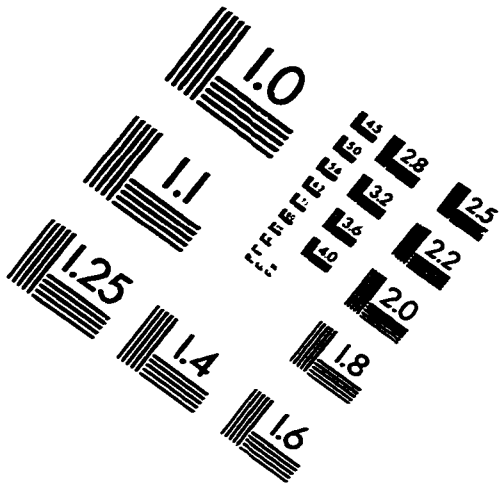

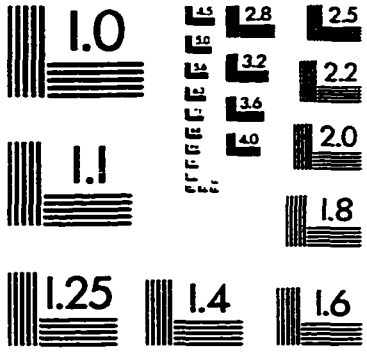

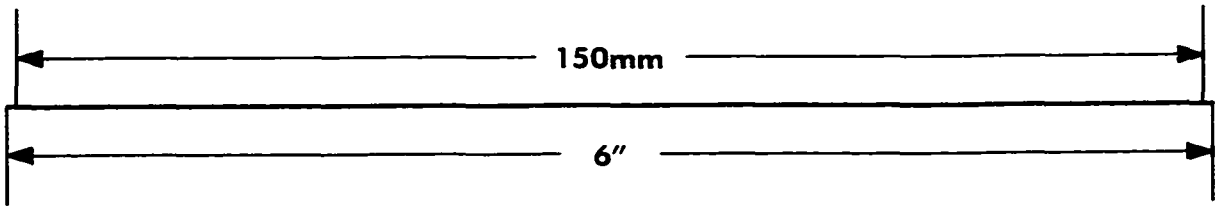

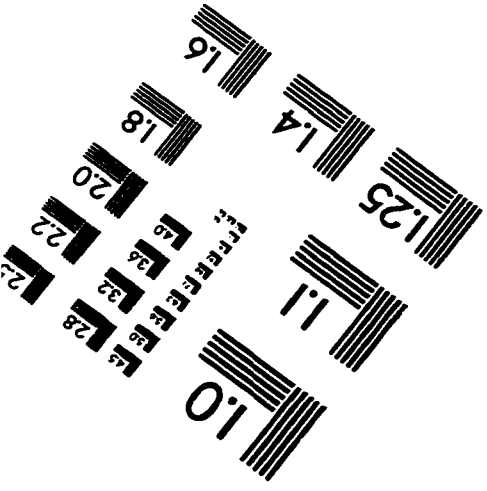

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