Aluminum-induced Nonenzymatic Phospho-incorporation into Human Tau and Other Proteins*

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Aluminum is one of the most abundant elements in the earth’s crust, and biological systems probably evolved in the presence of appreciable concentrations (1). The unavoidable exposure to aluminum is subject to considerable quantitative fluctuations resulting from variations in geographic, technological, cultural, medical, and other factors (2). Aluminum appears to be closely associated with several adverse neurological and skeletal effects (3). In particular, it has been suggested that elevated levels of aluminum in brain tissues correlate with the occurrence of neurological brain disorders such as Alzheimer’s disease, Down’s Syndrome, and Parkinsonism dementia (4-7). However, controversy exists over whether aluminum has a role in the etiology of Alzheimer’s disease (12). The presence of aluminum in neurofibrillary tangles, neurofibrillary degeneration, and in Alzheimer’s disease patients (13) has been associated with neurofibrillary tangle formation, and may thus represent a pathogenetic factor for the development of Alzheimer’s disease (13). The neurofibrillary tangles are composed of paired helical filaments whose main constituent is tau, a protein that is normally associated with microtubules (38). Tau contains multiple phosphorylation sites (39-41), can be phosphorylated in vitro by various protein kinases (42-45), and its function in vivo may be controlled, at least in part, by phosphorylation and dephosphorylation reactions (46-48). Tau in Alzheimer’s paired helical filaments is hyperphosphorylated at multiple sites and may undergo other modifications that result in the appearance of immunologically distinct forms (48-51). While hyperphosphorylation of tau appears to be a distinguishing criterion of Alzheimer’s disease (48), it is not known if this plays a causal role in the disease etiology. These observations suggest the possibility that aluminum may play some role in neurofibrillary tangle formation by acting on tau. To explore this hypothesis, we began investigating the ability of aluminum to potentiase the phosphorylation of human tau by a variety of protein kinases. In the course of these studies, we discovered that aluminum alone, without any kinase, could modify human tau in two distinct ways that mimic modifications that in vivo. It has been demonstrated that aluminum salts can induce a variety of physiological effects in culture and animal systems. For example, in vivo aluminum treatment can: 1) alter neurofilament protein phosphorylation (19-21); 2) induce cytoskeletal changes in human neural cultures (22, 23); 3) reduce expression of mRNA coding for neurofilament subunits (24); 4) induce expression of an immunological epitope associated with Alzheimer’s disease on human neuroblastoma cells (25, 26); and 5) induce neurofibrillary tangles in cultured rat neurons (13, 27, 28). These in vivo effects may be related to one or more of the biochemical effects of aluminum in vitro. These include its ability to: 1) promote tubulin polymerization (29); 2) promote the aggregation (30) and alter the electrophoretic properties (31) of highly phosphorylated neurofilament subunits from brain extracts; 3) inhibit dephosphorylation of tau protein in synaptosomal cytosol fractions (32); and 4) as aluminum fluoride, to activate G proteins’ and modulate adenylyl cyclase activity (33-35).

Aluminum is transported in the blood by binding to human serum transferrin and serum albumin (36), and it can be concentrated in neural tissues at surprisingly high levels, particularly in Alzheimer’s disease patients. Al³⁺ has been found at 0-0.6 ppm (dry weight) in normal human brain; in brains from Alzheimer’s patients it has been found at 0.5 ppm in nondegenerated regions and at 6-12 ppm in the areas of neurofibrillary degeneration (37). The presence of Al³⁺ in neurons is associated with neurofibrillary tangle formation, and may thus represent a pathogenetic factor for the development of Alzheimer’s disease (13). The neurofibrillary tangles are composed of paired helical filaments whose main constituent is tau, a protein that is normally associated with microtubules (38). Tau contains multiple phosphorylation sites (39-41), can be phosphorylated in vitro by various protein kinases (42-45), and its function in vivo may be controlled, at least in part, by phosphorylation and dephosphorylation reactions (46-48). Tau in Alzheimer’s paired helical filaments is hyperphosphorylated at multiple sites and may undergo other modifications that result in the appearance of immunologically distinct forms (48-51). While hyperphosphorylation of tau appears to be a distinguishing criterion of Alzheimer’s disease (48), it is not known if this plays a causal role in the disease etiology. These observations suggest the possibility that aluminum may play some role in neurofibrillary tangle formation by acting on tau. To explore this hypothesis, we began investigating the ability of aluminum to potentiate the phosphorylation of human tau by a variety of protein kinases. In the course of these studies, we discovered that aluminum alone, without any kinase, could modify human tau in two distinct ways that mimic modifications that

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1 The abbreviations used are: G protein, guanine nucleotide-binding protein; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
occur in Alzheimer’s disease: 1) aluminum induces aggregation of human tau (and other proteins); and 2) aluminum catalyzes nonenzymatic covalent transfer of the triphosphate group from ATP to tau.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, histone 1 (type III), β-casein from bovine milk, rabbit muscle enolase, and diithiothreitol were from Sigma. [γ-32P]ATP, [α-32P]ATP, and [2,8,5'-3H]ATP from Du Pont-New England Nuclear; [γ-32P]GTP and [α-32P]GTP from Amersham; [ε-32P]CTP from ICN; calf intestinal alkaline phosphatase from Boehringer Mannheim; bacterial alkaline phosphatase from Worthington; and aluminum chloride from Allied Chemicals.

**Purification of Human Tau—Escherichia coli carrying plasmid pET-N1234C was grown and induced with isopropyl-1-thio-β-D-galactopyranoside. Tau was purified from Triton X-100 lysates by sequential DE52 (Whatman) and P11 (Whatman) ion-exchange chromatography as described (52).**

**Tau Aggregation Assays**—10% SDS-PAGE was performed according to Laemmli et al. (53). For filtration assays, samples were loaded into wells of a Hoefer PR6000 Slot Blot apparatus equipped with a Nylon-Plus 0.45-μm membrane, and filtered under vacuum with two washes of 200 μl of deionized water. Protein amounts in the collected filtrates were assayed by the method of Bradford (54).

**Phospho-incorporation Assay**—Assays were performed in a final volume of 50 μl of 20 mM Hepes (pH 7.4), 1 mM DTT, 50 μM [γ-32P] ATP (2000-3000 cpm/pmol) containing 3 pg of purified tau or other indicated proteins; and AlCl3 from Allied Chemicals. ATP concentration was adjusted in the reaction assay to 100 μM using a stock solution of 1 M MgCl2 for 2 h at 37 °C.

**Alkaline Phosphatase Treatment**—Resuspended aliquots of γ-32P-labeled tau (3 μg each) were incubated with calf intestine or E. coli alkaline phosphatase is 60 μl of 50 mM Tris-Cl (pH 8.0), 15 mM MgCl2 for 2 h at 37 °C.

**Double Labeling Assay**—Assays were performed in a final volume of 50 μl of 20 mM Hepes (pH 7.4), 1 mM DTT, 0.8 μg of purified tau, [2,8,5'-3H]ATP (1.9 × 104 cpm/mol), and/or [α-32P]ATP (2.2 × 104 cpm/mol), without and with 500 μM AlCl3. ATP concentration was adjusted in the reaction assay to 100 μM using a stock solution of 1 mM cold ATP. Following 2 h incubation at 30 °C, reactions were stopped by adding 12.5 μl of 5 × SDS sample buffer and then adding 7 μl of 500 mM EDTA. Samples were incubated at 95 °C for 5 min and then loaded onto 10% SDS-PAGE gels.

**Alkaline Phosphatase Precipitation of 3'P-Labeled Tau**—3'P-Labeled tau was precipitated by addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid, 10 mM sodium pyrophosphate. After 1 h at 0 °C, the mixture was centrifuged in an Eppendorf microcentrifuge at maximum speed for 15 min. The precipitate was washed twice with the precipitating solution and twice with ice-cold 1:1 ethanol/ether (v/v). Precipitated tau was suspended in 20 mM Hepes (pH 7.4), divided into aliquots, and stored at −70 °C.

**RESULTS**

**A1P3 Induces Protein Aggregation**—Purified human tau was incubated with varying concentrations of AlCl3 for 30 min at 30 °C, analyzed by SDS-PAGE, and visualized by Coomassie Blue staining (Fig. 1). As previously observed,2 tau migrated with a relative mobility of M, ~50,000 even though its actual molecular weight is ~42,000. When tau was preincubated with aluminum, progressively reduced amounts were observed to enter the gel, particularly at concentrations above 100 μM. This effect of aluminum could be reversed by adding EDTA to 50 mM before electrophoresis (lane 7). In some experiments, a fraction of the tau which had been incubated with 300–500 μM aluminum was observed to migrate as a smear with retarded electrophoretic mobility (data not shown).

* R. Brandt and G. Lee, personal communication.

**Effect of preincubation with AlCl3 on entry of proteins into 10% SDS-polycrylamide gels**

Experiments like that shown in Fig. 1 were conducted with the indicated proteins. The optical densities of the Coomassie Blue-stained protein bands were estimated by computer-assisted video scanning (Bioimage Visage 110; Millipore Corp.) to calculate the amounts of protein entering the gel. All values are normalized to the amount entering following a 30-min incubation with buffer alone. Values are averages of two experiments; standard deviations were <5%.

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction of protein entering gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human tau</td>
<td>Bovine serum albumin β-Casein Enolase</td>
</tr>
<tr>
<td>30-min buffer only</td>
<td>1.0</td>
</tr>
<tr>
<td>30 min, 1 mM AlCl3</td>
<td>0.09</td>
</tr>
<tr>
<td>30 min, 1 mM AlCl3 then + 50 mM EDTA</td>
<td>0.8</td>
</tr>
<tr>
<td>24 h, 1 mM AlCl3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>24 h, 1 mM AlCl3 then + 50 mM EDTA</td>
<td>0.6</td>
</tr>
</tbody>
</table>

These phenomena suggested that aluminum was causing tau to aggregate.

Similar results were observed in experiments with bovine serum albumin, β-casein, and enolase (Table I). As with tau, the effect of 30 min incubation with aluminum could be reversed by subsequent addition of EDTA to 50 mM. However, when the proteins were preincubated with AlCl3 for 24 h, subsequent addition of EDTA only permitted tau, and not the other proteins, to enter the gels. The aggregation phenomenon was substantiated by measuring the effect of incubation with aluminum on the ability of tau and other proteins to pass through nylon membranes with 0.45-μm pores by filtration under pressure as described under “Experimental Procedures.” Consistent with the electrophoretic analyses, incubation with increasing concentrations of aluminum caused progressive reductions in passage through the membrane (Table II).

**Aluminum Induces Stable Phospho-incorporation into Tau**—Pilot experiments were conducted to test the effect of preincubation with aluminum on the ability of a variety of kinases to phosphorylate tau. In the process, we were surprised to discover that purified human tau was radioactively labeled to a significant extent when incubated for 30 min, 30 °C, with AlCl3 and [γ-32P]ATP alone. To exclude the possibility that the tau preparations contained any enzymatic kinase activity, experiments were performed using preparations which had been incubated at 95 °C for 10 min prior to the incubation with aluminum and ATP. Similar results were obtained with this heat-denatured tau preparation (Fig. 2).
Passage (normalized to 1 for the relative comparisons) was observed and filtered under vacuum as described under "Experimental Procedures." The relative amounts of protein in the filtrates were determined by the method of Bradford (54). For all proteins, 75-78% passage (normalized to 1 for the relative comparisons) was observed for samples treated with buffer containing no AlCl₃. Values are averages of two experiments; standard deviations were <10%.

**TABLE II**

Filtration of tau and other proteins through 0.45 μm nylon membranes following incubation with aluminum

<table>
<thead>
<tr>
<th>[AlCl₃] (μM)</th>
<th>Human tau</th>
<th>Bovine serum albumin</th>
<th>β-Casein</th>
<th>Enolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>50</td>
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<td>0.78</td>
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<tr>
<td>100</td>
<td>0.63</td>
<td>0.63</td>
<td>0.50</td>
<td>0.62</td>
</tr>
<tr>
<td>300</td>
<td>0.33</td>
<td>0.26</td>
<td>0.30</td>
<td>0.33</td>
</tr>
<tr>
<td>500</td>
<td>0.11</td>
<td>0.07</td>
<td>0.08</td>
<td>0.19</td>
</tr>
<tr>
<td>1000</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**Fig. 2.** Nonenzymatic phospho-incorporation into human tau by [γ⁻³²P]ATP and aluminum. Purified human tau (3 μg) was incubated in 50 μl of 20 mM Hepes (pH 7.4), 1 mM DTT, and 50 μM [γ⁻³²P]ATP (2,000-3,000 cpm/pmol) for 30 min, 30 °C, with different concentrations of AlCl₃ (lanes: 1, 0 μM; 2, 100 μM; 3, 300 μM; 4, 500 μM). After the incubation, EDTA was added to a final concentration of 50 mM and samples were analyzed by 10% SDS-PAGE, autoradiography, and scintillation counting. Coomassie Blue staining of the gel (not shown) indicated that essentially the same amount of tau was present in each lane. The stoichiometry of labeling in lane 4 was about 0.20 mol of ³²P/mol of tau.

**Fig. 3.** Nonenzymatic phospho-incorporation into human tau by [γ⁻³²P]GTP or [α⁻³²P]GTP and aluminum. Purified human tau was incubated with 50 μM [γ⁻³²P]GTP (2,000-3,000 cpm/pmol) (lanes 1-6) or 50 μM [α⁻³²P]GTP (2,000-3,000 cpm/pmol) (lanes 7-12) and varying concentrations of AlCl₃ (lanes 1 and 7, 0 μM; 2 and 8, 50 μM; 3 and 9, 100 μM; 4 and 10, 300 μM; 5 and 11, 500 μM; 6 and 12, 1 mM) and analyzed by SDS-PAGE and autoradiography as described in the legend to Fig. 2. Coomassie Blue staining of the gel (not shown) indicated that essentially the same amount of tau was present in each lane.

Only trace labeling was observed with [γ⁻³²P]ATP without AlCl₃; this increased markedly with increasing concentrations of AlCl₃ to a maximum stoichiometry of labeling of about 0.2 mol of ³²P/mol of tau at about 500 μM Al³⁺. Similar results were obtained with [γ⁻³²P]GTP (Fig. 3), but negligible association was observed with [³²P]orthophosphate (data not shown).

In additional investigations, we found that aluminum sulfate and aluminum nitrate were as effective as aluminum chloride for inducing labeling of tau. On the other hand, aluminum tartarate did not cause aggregation or phospho-incorporation at all (Fig. 4). This probably indicates that free AICl₃ is required for this phenomenon: unlike the other salts, aluminum tartarate has a poor ability to dissociate and release Al³⁺ ions.

The stability of the association between ³²P and tau during SDS-PAGE suggested that the linkage was covalent. To verify this, tau which had been preincubated with [γ⁻³²P]ATP and 500 μM AlCl₃ and then precipitated by trichloroacetic acid (designated γ⁻τau) was analyzed by SDS-PAGE. Greater than 70% of the ³²P radioactivity was stably associated with γ⁻tau, even after extensive washing with 2 N NaCl (which is expected to dissociate any ionic bonds). To distinguish this incorporation from the conventional (enzyme catalyzed) transfer of the γ-phosphate alone, we refer to it as "phospho-incorporation."

**Aluminum-catalyzed Phospho-incorporation into Other Proteins—**The abilities of varying concentrations of AlCl₃ to induce nonenzymatic phospho-incorporation into β-casein, histone, and enolase were tested in experiments like that shown in Fig. 2. In agreement with previous reports (55, 56), some amount of nonenzymatic ³²P labeling of histone and enolase was observed in the absence of AlCl₃ (Fig. 5). However, in contrast with the results observed with tau, the
presence of aluminum did not enhance this phenomenon; in fact, aluminum decreased the labeling of histone. AlCl₃ did stimulate phospho-incorporation into β-casein, although the stoichiometry was slightly lower than that observed with tau (i.e. 0.22 ± 0.04 for tau and 0.16 ± 0.01 for β-casein).

**Nature of Chemical Linkage between ³²P and Tau**—To investigate the type of covalent linkage between the ³²P and tau catalyzed by Al³⁺, experiments like that shown in Figs. 2 and 3 with γ-³²P-nucleotides were also conducted using [α-³²P]ATP, [α-³²P]GTP, and [α-³²P]CTP (Fig. 3 and data not shown). Increasing concentrations of AlCl₃ caused a dramatic enhancement in phospho-incorporation like that observed with γ-³²P ATP. Labeling by the α-³²P-nucleotides indicated that the phospho-incorporation reaction is not a standard phosphorylation reaction in which only the γ-phosphate is transferred, and that at least the entire triphosphate moiety was linked to tau.

To determine whether only the triphosphate group or whether other portions of the ATP molecule were linked to tau, double labeling experiments were performed in which human tau protein was incubated with [2,8,5',³-H]ATP (i.e. labeled both within the base and sugar moieties of the ATP) and/or [α-³²P]ATP in the presence and absence of 500 μM Al³⁺ in experiments like those shown in Figs. 2 and 3. Covalent linkage of the sugar and base moieties of ATP (detected by ³H labeling) was negligible compared with incorporation of the phosphate moiety (detected by ³²P labeling) (Table III). This suggested that the phospho-incorporation reaction was actually a "triphosphorylation" reaction in which the entire triphosphate group, but not the sugar or base, was transferred to tau.

This hypothesis was consistent with experiments showing that the ³²P could be removed from γ'-tau by either calf intestinal alkaline phosphatase or *E. coli* alkaline phosphatase (Fig. 6). Since these phosphatases interact specifically with monosubstituted phosphatase, this implied that either: 1) the γ-phosphate was not directly linked to tau (and thus is directly accessible to the phosphatase); or 2) if the γ-phosphate was directly linked, that only the triphosphate moiety from the ATP was transferred to tau (so that the phosphatase could act directly on the α-phosphate and subsequently on the β- and γ-phosphates).

The first possibility was supported by an experiment indicating that the linkage of the γ-phosphate to tau is more labile than that of the α-phosphate (57). Both trichloroacetic acid-precipitated α'-tau and γ'-tau without phosphatase treatment. [α-³²P]ATP (α'-tau) were partially hydrolyzed in 1 N HCl (10 min, 95 °C) and the remaining tau-associated radioactivities were measured by scintillation counting following subsequent trichloroacetic acid reprecipitation. Over 70% of the ³²P in α'-tau was resistant to this treatment, but only 13% was resistant in γ'-tau (data not shown).

Trichloroacetic acid-precipitated γ'-tau was not affected by incubation with either 0.1 N HCl for 60 min or 0.2 N hydroxylamine for 30 min at 37 °C (Fig. 7). In contrast, most of the ³²P was lost after treatment with 0.1 N NaOH for 60 min at 37 °C. The stability of the linkage under acid and hydroxylamine treatment indicates that the phosphorilation does not represent N-linkage to arginine, histidine, or lysine (58, 59), nor acyl-linkage to aspartate or glutamate (59). Its partial lability following alkali treatment is consistent with O-linkage to serine, threonine, and/or tyrosine (59).

**Effects of Other Di- and Trivalent Metals on Tau**—To acquire some insight into the specificity of Al³⁺ for these effects, we studied the effects of preincubating tau with 500 μM of various cations (Cd²⁺, Co²⁺, Cu²⁺, Hg²⁺, Mn²⁺, Sn²⁺, Sr²⁺, Zn²⁺, Al³⁺, Fe³⁺, Sc³⁺, Sn³⁺, and Zr⁴⁺) on its entry into SDS-PAGE. None of the eight divalent or the two quadrivalent cations had any significant effect. However, all three trivalent cations (aluminum, ferric, and scandium) aggregated human tau induced nonenzymatic labeling by γ-³²P ATP in the ratios 1:0.3:2, respectively.

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**Table III**

<table>
<thead>
<tr>
<th>Type of labeled ATP</th>
<th>-Al³⁺</th>
<th>+Al³⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2,8,5'-³-H]ATP</td>
<td>≤0.001</td>
<td>≤0.001</td>
</tr>
<tr>
<td>[2,8,5'-³-H]ATP</td>
<td>≤0.001</td>
<td>≤0.001</td>
</tr>
<tr>
<td>[α-³²P]ATP</td>
<td>0.015 ± 0.003</td>
<td>0.11 ± 0.002</td>
</tr>
<tr>
<td>[α-³²P]ATP</td>
<td>0.018 ± 0.004</td>
<td>0.14 ± 0.01</td>
</tr>
</tbody>
</table>

**FIG. 6. Dephosphorylation of γ'-tau by alkaline phosphatases.** Human tau which had been labeled by incubation with [γ-³²P] ATP and 500 μM AlCl₃ as described in the legend to Fig. 2 was trichloroacetic acid precipitated, resuspended, and incubated with either calf intestine alkaline phosphatase (lane 2, 0.025 units; lane 3, 0.06 units; lane 4, 0.14 units; lane 5, 0.29 units) or 0.04 units of *E. coli* alkaline phosphatase (lane 6) as described under "Experimental Procedures." Samples were analyzed by 10% SDS-PAGE and autoradiography. Lane 1, γ'-tau without phosphatase treatment. Lanes 7 and 8 display γ'-tau after incubation with 0.29 units of heat-denatured calf intestine alkaline phosphatase or 0.18 units of *E. coli* alkaline phosphatase, respectively. Coomassie Blue staining of the gel (not shown) indicated that essentially the same amount of tau was present in each lane.

**FIG. 7. Susceptibility of human tau phospho-incorporation to chemical treatment.** Purified human tau was labeled with [γ-³²P] ATP in the presence of 500 μM AlCl₃ and precipitated with trichloroacetic acid as described under "Experimental Procedures." The precipitated tau was resuspended in 20 mM Hepes (pH 7.4), divided into four aliquots, and was incubated with: no addition (lane 1), 0.1 N HCl for 60 min (lane 2), 0.1 N NaOH for 60 min (lane 3), or 0.2 M hydroxylamine at pH 7.5 for 30 min (lane 4), all at 37 °C, and then analyzed by 10% SDS-PAGE and autoradiography.
DISCUSSION

The role of aluminum in neuron degeneration has been extensively investigated ever since increased aluminum content was detected in areas of human brain containing neurofibrillary degeneration (4). Epidemiologic analysis has linked increased incidence of Alzheimer’s disease to relatively higher concentrations of aluminum in water (60), but biochemical mechanisms for a potential causal role of aluminum in Alzheimer’s disease are unknown.

This study indicates that aluminum induces aggregation of tau, which prevents its entry into SDS-polyacrylamide gels at [Al] > 100 μM. Similar effects of Al have been reported with other neurofilament proteins (30, 31, 61). However, aggregated neurofilament proteins did not recover normal electrophoretic mobility following treatment with EDTA (31) while our experiments show that 50 mM EDTA reverses the effect of Al on tau. Aluminum-induced aggregation of tau was not dependent on the presence of SDS as was also observed in a SDS-independent membrane filtration assay. It has been suggested that aluminum-induced aggregation of brain cytoskeletal proteins results from association of the Al-positive charge with negatively charged protein-linked phosphates (30, 31, 61, 62). However, this did not appear to be the case in our experiments: we found that prior incubation of tau with calf intestine alkaline phosphatase (10 times more than the amount used in the dephosphorylation experiment of Fig. 6) had no noticeable effect on the ability of Al to block entry of tau into SDS-polyacrylamide gels (data not shown). The aggregation effect was not specific to tau and neurofilament proteins: similar effects were observed with unrelated acidic and basic proteins.

We were surprised to discover that aluminum can induce nonenzymatic catalysis of a covalent linkage that resulted in the incorporation of the α- and γ-phosphates of ATP, GTP, and CTP into human tau. It is highly unlikely that the linkage merely represents noncovalent trapping because it was stable during electrophoresis in 0.1% SDS as well as subsequent treatment with 2 N NaCl and trichloroacetic acid precipitation. The fact that aluminum tartsarate, a tightly bound organic salt of aluminum, had no effect suggests that free Al is needed for both the nonenzymatic phospho-incorporation and aggregation phenomena.

The fact that both the α- and γ-phosphates were linked to tau indicates that the reaction is not a simple phosphorylation involving transfer of the γ-phosphate alone, but that the entire triphosphate is linked to the protein. The double labeling experiment, in which the phosphate and nucleoside moieties were labeled with different isotopes, clearly indicated that only the triphosphate group was transferred. Furthermore, the rapid loss of radioactivity from γ-tau relative to the rate of loss from α-tau after treatment with 1 N HCl at 95 °C or with alkaline phosphatase suggests that the linkage is via the α-phosphate, not the γ-phosphate. This probably accounts for the observation that somewhat more radioactive labeling was observed with [α-32P]GTP than with [γ-32P]GTP (possibly due to the effects of contaminating phosphatases which would act first on the γ-phosphate) (Fig. 5).

The chemical stability experiments (Fig. 7) suggest that the triphosphorylation is via N-linkage to serines, threonines, and/or tyrosines. A relatively direct aluminum-catalyzed transfer of the α-phosphate from the hydroxyl of the nucleotide sugar to a hydroxyl of the protein, while a novel reaction, seems plausible. Clearly, further experimentation will be needed to confirm this triphosphorylation hypothesis.

Nonenzymatic covalent modifications of proteins by nucleotides have been reported (55). However, the aluminum-catalyzed phospho-incorporation reaction is clearly distinct from the reported divalent cation-induced phosphorylation of tyrosine (56); this nonenzymatic phosphorylation is most efficiently stimulated by Mn2+, an ion with no apparent effect in our assay. It is also evident that phospho-incorporation is different from the nonenzymatic nucleotidylation of platelet-derived endothelial growth factor which required Mn2+, SDS, reducing agents, and incubation at 95 °C (63).

The ability of Al to catalyze triphosphorylation may reflect that fact that it binds ATP about 10 times more strongly than does Mg2+ (64); coordination of Al with the negatively charged oxygens of the ATP may be involved in the catalysis. Other trivalent cations also induced both aggregation and phospho-incorporation. While iron induced phospho-incorporation only weakly, scandium was even more effective than aluminum. However, scandium is 103 times less abundant in the environment than aluminum and high concentrations have not been reported in body tissues so it is unlikely that it plays a physiological role (65). In contrast, the 12 ppm (dry weight) aluminum concentrations that have been observed in Alzheimer’s patients’ brains (13) correspond to 90 μM (assuming that neurons contain ~80% water) and are in the range required for the effects observed here.

The high concentration of aluminum in neurofibrillary tangles in Alzheimer’s disease (18) is consistent with the hypothesis that aluminum plays a causal role in tangle formation. Interestingly, aluminum concentrations are not elevated in β-amyloid plaques (16), another histological hallmark of Alzheimer’s disease, suggesting that any etiologic role of aluminum is confined to tangle formation. It is possible that aluminum cross-bridges might promote the aggregation of tau into paired helical filaments of definite structure (66). This phenomenon might be promoted by aluminum cross-bridges. If so, it will be important to determine why only tau, and not other proteins, are aggregated at those locations in the brain containing high aluminum concentrations. Factors governing aluminum transport and sequestration could obviously play an important role.

Although we focussed particularly on the effects of aluminum on tau, other proteins can also be similarly affected in vitro and might possibly be affected in vivo. Thus, another triphosphorylation might be involved in the aluminum-induced increase in the incorporation of phosphatase into microtubule-associated and neurofilament proteins in rat brains following administration of aluminum in their drinking water (21); it could also be involved in the induction of an antigenic epitope associated with Alzheimer’s disease neurofibrillary tangles that has been observed after aluminum treatment of human neuroblastoma cells (25). In many of these studies it is not clear whether reports of protein phosphorylation actually refer to attachment of a single phosphate to amino acid side chains or if other modifications such as triphosphorylation have occurred. New methods for detecting these novel modifications in proteins from nonradioactive tissue samples are needed. It will be important to determine the exact nature of the covalent modification of tau catalyzed by aluminum and to see if it occurs in normal or diseased human brain.

Acknowledgments—This study emanated from ideas originally suggested by Efraim Racker. We thank Gloria Lee and Roland Brendt for tau-expression plasmid pET-N1234C, Marwan El-Sabbab for computer-assisted densitometry, and Rick Cerione, Leon Heppel, Virginia Lee, and David Usher for helpful conversations.

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