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Research Article

Inactivation of human glutamate dehydrogenase by aluminum

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Abstract. Aluminum inactivated glutamate dehydrogenase (GDH) by a pseudo-first-order reaction at micromolar concentrations. A double-reciprocal plot gave a straight line with a k_{inact} of 2.7 min^{-1} and indicated the presence of a binding step prior to inactivation. The inactivation was strictly pH dependent and a marked increase in sensitivity to aluminum was observed as the pH decreased. At a pH higher than 8.5, no inactivation was observed. The completely inactivated GDH contained 2 mol of aluminum per mole of enzyme subunit monomer. When preincubated with enzyme, several chelators such as citrate, NaF, N-(2-hydroxyethyl) ethylenediaminetriacetic acid or ethylenediaminetriacetic acid efficiently protected the enzyme against the aluminum inactivation. In a related experiment, only citrate and NaF released the aluminum from the completely inactivated aluminum-enzyme complex and fully recovered the enzyme activity. Ferritin, $NADP^+$, or nerve growth factor did not show any effects on the recovery of the aluminum-inactivated GDH activity. The dissociation constant for the aluminum-enzyme complex was calculated to be 5.3 μM. Although aluminum has been known to form a complex with nucleotides, no such effects were observed in the inactivation of GDH by aluminum as determined using GDHs mutated at the ADP-binding site, NAD^+-binding site or GTP-binding site. Circular dichroism studies showed that the binding of aluminum to the enzyme induced a decrease in α helices and β sheets and an increase in random coil. Therefore, inactivation of GDH by aluminum is suggested to be due to the conformational change induced by aluminum binding. These results suggest a possibility that aluminum-induced alterations in enzymes of the glutamate system may be one of the causes of aluminum-induced neurotoxicity.

Key words. Glutamate dehydrogenase; aluminum; glutamate; chelator; conformational change.

Many studies have investigated the involvement of aluminum in diverse cellular functions including progression of neurodegeneration. For example, aluminum can induce the production of tumor necrosis factor-α in human glioblastoma cells [1] and it can induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of gadd 153 and NF-κB [2]. Intracisternal injection of aluminum complexes into rabbit brain induces cytochrome c release from mitochondria, a decrease in Bcl-2 in both mitochondria and endoplasmic reticulum, Bax translocation into mitochondria, activation of caspase-3 and DNA fragmentation [2]. Aluminum also affects the production of nerve growth factor (NGF) in mice [3]. Very recently, the effects of aluminum on the proinflammatory cytokine/neurotrophin balance in cultured neuronal cells have been reported [4].

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Aluminum is a strong inhibitor of some enzymes of the glycolysis pathway such as hexokinase [5] and phosphofructokinase [6]. Aluminum also affects several enzymes such as acetylcholinesterase [7], monoamine oxidase type B [8], dihydropterine reductase [9], α-chymotrypsin [10], carbonic anhydrase and Na^+,K^+-ATPase [11]. Most recent studies have shown that aluminum administration stimulates superoxide dismutase activity and glutathione peroxidase activity and decreases glutathione S-transferase in various brain regions, providing evidence that oxidative stress is involved in aluminum toxicity to the brain [12, 13].

Alteration of the glutamate system has been postulated to be involved in aluminum-induced neurotoxicity [14–16]. Aluminum affects synaptosomal uptake systems with some selectivity toward the uptake of glutamate [17, 18]. Glutamate is a potential binder of aluminum in physiological solutions and aluminum can cross the blood-brain barrier as a glutamate complex [19, 20]. In vivo aluminum exposure significantly enhanced glutamate levels in the cerebrum, thalamic area, midbrain hippocampal region and cerebellum as well as alterating activities of associated enzymes in glutamate metabolism [21–23]. One central enzyme in glutamate metabolism is glutamate dehydrogenase (GDH). GDH is a family of enzymes, catalyzing a reversible deamination of L-glutamate to α-ketoglutarate directly connected to the Krebs cycle. Therefore, GDH serves as the major link between carbohydrate and amino acid metabolism and various roles for GDH in the central nervous system have been reported. For instance, partial deficiency of GDH isozymes has been reported in some patients with cerebellar degeneration, suggesting that the enzymes are important in brain function [24, 25]. Reduced GDH activity has also been reported in patients with Alzheimer's disease and Parkinson's disease [26]. In addition, GDH has shown neuroprotective value in model systems where glutamate reuptake is inhibited [27]. Inhibition of GDH expression by antisense oligonucleotides has also been reported to be toxic to cultured mesencephalic neurons, with dopaminergic neurons being affected at the early stages of this inhibition [28]. In addition, Cavallaro et al. [29] have identified GDH as one of the late memory-related genes in the hippocampus, and Dutuit et al. [30] have observed a specific alteration in the expression of GDH in rats with genetic absence epilepsy.

Aluminum has been reported to play a very important role in reducing the activity of Krebs cycle enzymes and GDH in rat brain homogenate [31]. Very recently, Yang et al. [32] reported that aluminum promotes α-ketoglutarate, a substrate of GDH, tautomerization to its enolic structure compounds in solution. At present, however, the reaction mechanism and a unified hypothesis to explain the effects of aluminum on the aluminum-induced alteration of enzymes in glutamate metabolism are not available. The purpose of this study was to determine the direct binding of aluminum to human GDH.

Materials and methods

Reagents

NADH, citric acid, transferrin (human), ferritin (equine spleen), ethylenediaminetriacetic acid (EDTA), N-(2-hydroxyethyl) ethylenediaminetriacetic acid (HEDTA), NaF, α-ketoglutarate and $AlCl_3$ were purchased from Sigma (St. Louis, Mo.). NGF (7S mouse submandibular glands) was purchased from Alomone Labs (Jerusalem, Israel). Aluminum standard solution was from Aldrich (Milwaukee, Wis.). A human GDH-encoding synthetic gene was expressed in *Escherichia coli* and over-expressed soluble recombinant human GDH was purified homogeneously as described before [33]. Cassette mutagenesis at Lys450, Tyr187 and Glu279 was performed separately to make mutant GDHs at the GTP-binding site (K450G), ADP-binding site (Y187G) and NAD^+-binding site (E279G), respectively, as described elsewhere [34–36]. Unless otherwise specified, highly purified enzymes were used for the studies. All other chemicals and solvents were reagent grade or better.

Measurement of aluminum

The concentration of aluminum ion was measured by an atomic absorption spectrophotometer according to a modification of the method of D'Haese et al. [37]. Pyrolytically coated graphite tubes and pyrolytical platforms were used with 10-µl aliquots. Before use, the tubes and platforms were conditioned by being heated twice at 2800 °C. The 309.2-nm aluminum wavelength, a 160-µm slit width and a lamp current of 8 mA were used. Background correction was performed throughout.

GDH assay and kinetics

GDH activity was measured spectrophotometrically in the direction of reductive amination of α-ketoglutarate by following the decrease in absorbance at 340 nm as described before [38] except that no ADP was used unless otherwise indicated. All assays were performed in triplicate and initial velocity data were correlated with a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 10 mM α-ketoglutarate and 0.1 mM NADH at 25 °C. One unit of enzyme was defined as the amount of enzyme required for oxidizing 1 µmol of NADH per minute at 25 °C. The apparent first-order rate constant of inhibition (k_{app}) was obtained for each concentration by following the time course of development of inhibition by aluminum chloride as described elsewhere [39]. K_M and k_{inact} values were calculated from the double-reciprocal plots of the apparent first-order constants of inhibition (k_{app}) against the

concentration of aluminum. Protein concentration was determined using Coomassie brilliant blue G250 (Sigma) as described before [38] and calibrated with a bovine liver GDH as standard.

Quantitation of aluminum binding to GDH

To determine the amount of aluminum bound to the enzyme in a typical experiment, GDH (5 µM) was incubated at 25 °C for 30 min with 50 µM $AlCl_3$ in 25 mM HEPES, pH 6.5 in a final volume of 0.2 ml. The completely inactivated enzyme was dialyzed three times against 2000 vol of 25 mM HEPES, pH 6.5, at 4 °C for 12 h and assayed for protein and aluminum. The dissociation constant of aluminum for the enzyme-metal ion complex (E-Al) was measured by an indirect method using NaF. The E-Al complex was prepared as described above and incubated with varying amounts of NaF. The Al-F complex was separated from E-Al by centrifugation for 10 min at 2900 rpm in Centrifree filters (Amicon). The aluminum concentration in the filtrate was used to measure the amount of aluminum removed from E-Al by NaF.

Circular dichroism study

Proteins were incubated with or without 50 µM $AlCl_3$ in 25 mM HEPES, pH 6.5 or pH 8.5 at 25 °C for 30 min and far-UV circular dichroism (CD) spectra were monitored from 200 to 250 nm in a cuvette of 1-mm path length, 50-mdeg sensitivity, response time of 1 s, and scan speed of 50 nm/min on a Jasco J-715 spectrophotometer. The spectra were recorded as a five-scan average value and analyzed by the methods of Yang et al. [40] reading from a smoothed curve through a five-successive-point window average assigned to the middle to determine the secondary conformation. After subtracting appropriate blanks, mean residue ellipticities were calculated, using the formula $\theta = \theta_{obs} \times MRW/10\ cl$, where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight, c is the concentration of protein in g/ml and l is the path length in centimeters [41]. A mean residue molecular weight 110 was used. The spectrum of the inactive enzyme-aluminum complex was compared to that of the control enzyme at each pH.

Results

Inactivation of GDH by aluminum

Preincubation of hGDH with $AlCl_3$ in 25 mM HEPES, pH 6.5 at 25 °C inactivated the enzyme. The loss of enzyme activity was proportional to the preincubation time and to the concentration of aluminum (fig. 1 A). Inactivation of hGDH was pseudo-first order at each fixed concentration of aluminum (fig. 1 A). In numerous experiments, more than 99% of the enzyme activity was abolished in this manner. The apparent first-order rate constant of inactivation (k_{app}) was obtained for each concentration. A double-reciprocal plot gave a straight line with a k_{inact} value of 2.7 min^{-1} and K_M value of 7.7 µM (fig. 1 B), indicating the presence of a binding step prior to inactivation. The inactivation of GDH by aluminum was strictly pH dependent. When the effect of $AlCl_3$ on GDH was examined at different pHs, a marked increase in sensitivity to aluminum was observed as the pH decreased (fig. 2). An inhibitory effect of $AlCl_3$ was predominant below pH 7.0. Above pH 8.0, $AlCl_3$ did not affect the rate of the enzymatic reaction.

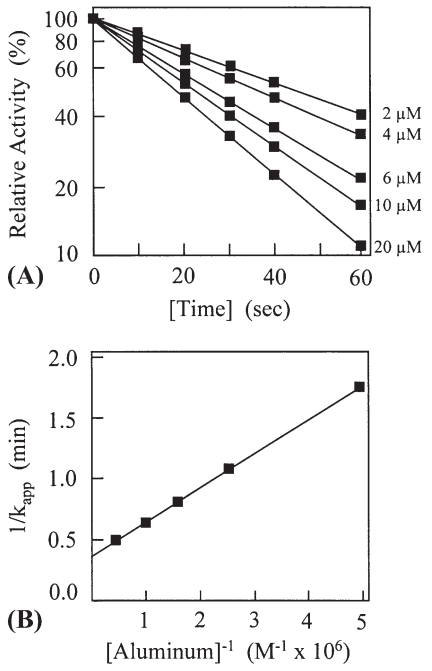

Figure 1. Inactivation of GDH by aluminum in pseudo-first-order reaction. (*A*) Time course of inactivation of GDH by $AlCl_3$. The enzyme was incubated with various concentrations of $AlCl_3$ in 25 mM HEPES, pH 6.5 at 25 °C. At the indicated times, the remaining enzyme activities were calculated. The apparent first-order rate constant of inhibition (k_{app}) was obtained for each concentration by following the time course of development of inhibition by aluminum and is expressed in a log plot. (*B*) Double-reciprocal plot of the apparent first-order rate constants of inactivation (k_{app}) against concentration of aluminum. K_M and k_{inact} values were calculated from the double-reciprocal plots of the apparent first-order constants of inhibition (k_{app}) against the concentration of aluminum.

Binding of aluminum to GDH

To quantitate the aluminum bound to the human GDH, an inactive enzyme-aluminum complex was prepared. GDH (1 µM) was incubated at 25 °C for 30 min with 50 µM $AlCl_3$ in 25 mM HEPES, pH 6.5 in a final volume of 1.0 ml and dialyzed three times against 2000 vol of 25 mM HEPES, pH 6.5 at 4 °C for 12 h. The protein-bound aluminum was measured using an atomic absorption spectrophotometer. The results showed that the com-

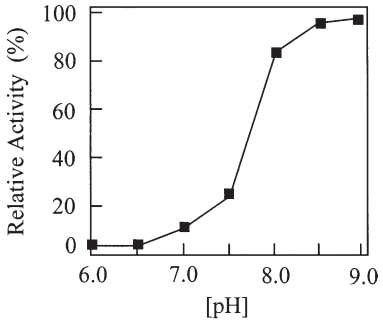

Figure 2. pH-dependent inactivation of GDH by $AlCl_3$. GDH was treated with or without 20 μM $AlCl_3$ in 25 mM HEPES at various pHs for 5 min at 25 °C. Remaining activities were measured and expressed as percentage of each control.

Table 1. Aluminum binding to the wild-type and mutant GDHs.

Treatment	[aluminum]/[subunit monomer]			
	wild-type	K450G	Y187G	E279G
Enzyme (control)	0.09 (0)	0.05 (0)	0.10 (0)	0.08 (0)
Enzyme + $AlCl_3$	1.98 (2)	1.89 (2)	2.05 (2)	2.11 (2)

Wild-type and mutant GDHs (5 μM) at the GTP-binding site (K450G), ADP-binding site (Y187G) or NAD^+-binding site (E279G) were treated with 50 μM $AlCl_3$ and dialyzed against 25 mM HEPES, pH 6.5 in a final volume of 0.2 ml. The contents of aluminum in the dialyzed samples were measured and expressed as moles of aluminum per mole of enzyme subunit monomer. The numbers in parentheses are the nearest integer values. Removal of the traces of aluminum from the control by dialysis against 0.1 mM citrate, pH 6.5 followed by removal of citrate against 25 mM HEPES, pH 6.5 did not affect the activity of the control enzyme.

pletely inactivated enzyme-aluminum complex contained 1.9 mol of aluminum per subunit (table 1). On the basis of an HPLC gel filtration, the molecular weight of the aluminum-inactivated GDH was similar to that of the native hexameric GDH (data not shown), suggesting that inactivation of GDH by aluminum is not due to the dissociation of the native enzyme. These results suggest that there are rather specific sites for the aluminum binding on the enzyme.

Formation of nucleic acid-aluminum complex

Aluminum is known to form a complex with nucleic acids [42] and mammalian GDHs are strictly regulated by GTP, ADP and NAD^+ [43]. Therefore, the possibility that aluminum may bind to GDH through the nucleotide-aluminum complex was examined. As shown in table 1, no such nucleotide-aluminum complexes were observed in the inactivation of human GDH by aluminum as determined using GDHs mutated at the GTP-binding site (K450G), ADP-binding site (Y187G) or NAD^+-binding site (E279G), these mutant GDHs being unable to bind

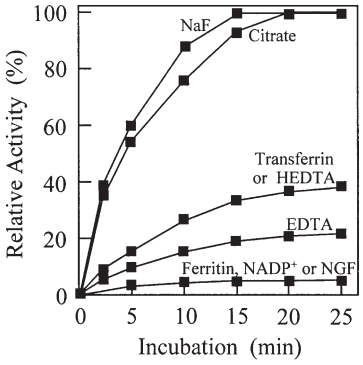

Figure 3. Reactivation of aluminum-inactivated GDH by chelators. GDH (5 μM) was incubated at 25 °C for 30 min with 50 μM $AlCl_3$ in 25 mM HEPES, pH 6.5 in a final volume of 0.2 ml and free aluminum was removed by dialysis three times against 2000 vol of 25 mM HEPES, pH 6.5, at 4 °C for 12 h. The completely inactivated enzyme was treated with several chelators (100 μM). At different times, aliquots were withdrawn and assayed for activity.

GTP, ADP or NAD^+, respectively [34–36]. When the wild-type and mutant GDHs were treated with 50 μM $AlCl_3$ and dialyzed as described above, there were no differences in the sensitivity to aluminum binding between the wild-type and mutant GDHs. All mutant GDHs still bound approximately 2 mol of aluminum per mole of enzyme subunit (table 1). These results indicate that aluminum binding to human GDH is not due to the nucleotide-aluminum complex and aluminum-binding sites are different from those of GTP, ADP and NAD^+.

Reactivation of GDH by chelators against aluminum inactivation

The results in figure 3 show that the inhibitory effect of aluminum is abolished by some chelators. GDH (5 μM) was incubated at 25 °C for 20 min with 50 μM $AlCl_3$ in 25 mM HEPES, pH 6.5 in a final volume of 0.2 ml and dialyzed three times against 2000 vol of 25 mM HEPES, pH 6.5, at 4 °C for 12 h. The completely inactivated enzyme was treated with several chelators (100 μM) such as citrate, NaF, HEDTA, EDTA, transferrin, ferritin, $NADP^+$ and NGF in a 0.1-ml final volume. At different times, aliquots were withdrawn and assayed for their activity in the standard assay mixture. Only citrate or NaF released the aluminum from the completely inactivated aluminum-enzyme complex and fully recovered the enzyme activity within 20 min. The effects of HEDTA, transferrin or EDTA on the reactivation of the aluminum-inactivated GDH were much weaker than those of NaF or citrate. Approximately 40% activity was recovered with transferrin or HEDTA and only 20% activity was recovered with EDTA. Ferritin, $NADP^+$ and NGF did not show any effects on the recovery of the aluminum-inactivated GDH activity.

In a related experiment, 50 μM $AlCl_3$ was preincubated with 2 mM of citrate, NaF, HEDTA, EDTA or $NADP^+$ for

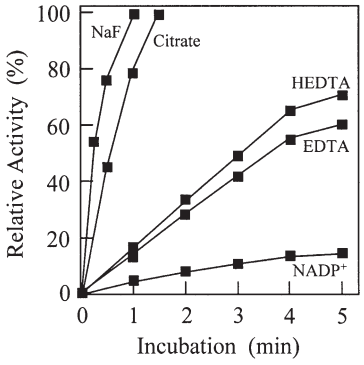

Figure 4. Protection of GDH by chelators against aluminum inactivation. $AlCl_3$ (50 µM) was preincubated with several chelators (2 mM) in 25 mM HEPES, pH 6.5 at 25 °C for various times. The reaction was started by the addition of enzyme and assay mixture.

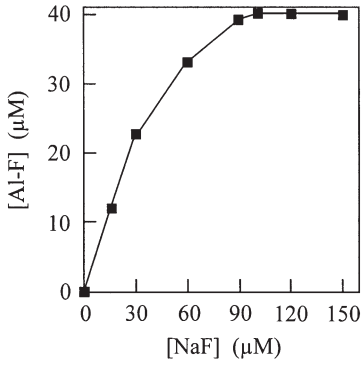

Figure 5. Removal of aluminum GDH-Al complex by NaF. Fluoride was used to remove aluminum from the enzyme-aluminum complex (E-Al). The completely inactivated enzyme (5 µM) was prepared as described above and incubated with varying amounts of NaF in 25 mM HEPES, pH 6.5 at 25 °C for 30 min in a 0.2 ml final volume. The aluminum-fluoride complex (Al-F) was separated from the E-Al by centrifugation for 5 min at 2900 rpm in Centrifree filters (Amicon). The aluminum concentration in the filtrate was used to measure the amount of aluminum removed from E-Al by NaF.

various times and then tested as a source of aluminum available to inhibit the enzyme. Only very small amounts of free aluminum seemed to have remained in solution in the presence of citrate or NaF, as shown by the full recovery of the enzyme activity within 2 min of incubation with these chelators (fig. 4). The results showed that the rate of chelation of aluminum by HEDTA or EDTA was slower than those of citrate or NaF because a significant amount of free aluminum was retained in solution to produce 25–40% inhibition even after 5 min (fig. 4). However, these chelating properties of HEDTA or EDTA for GDH activity against aluminum inactivation were much more effective than those in the reactivation experiments shown in figure 3, suggesting that HEDTA or EDTA have a better chelating power when directly interacted with aluminum prior to the formation of the aluminum-enzyme complex. $NADP^+$ showed the slowest rate of chelation of aluminum because more than 80% inactivation was still observed (fig. 4).

Measurement of dissociation constant for GDH-aluminum complex

The dissociation constant for the aluminum-enzyme complex was calculated by an indirect method using NaF, a known reversible chelator for aluminum [44, 45]. Accordingly,

$$\text{E-Al} \leftrightarrow \text{Al} + \text{E} \qquad {}^{Al}K_d(\text{E-Al}) = [\text{Al}][\text{E}]/[\text{E-Al}] \quad (1)$$

$$\text{F} + \text{Al} \leftrightarrow \text{Al-F} \qquad {}^{Al}K_a(\text{F}) = [\text{Al-F}]/[\text{Al}][\text{F}] \quad (2)$$

$$\text{sum: E-Al} + \text{F} \leftrightarrow \text{E} + \text{Al-F} \qquad K_{eq} = [\text{E}][\text{Al-F}]/[\text{E-Al}][\text{F}] \quad (3)$$

Here [E], [Al] and [F] are unliganded forms. From these three reactions,

$$K_{eq} = [\text{E}][\text{Al-F}]/[\text{E-Al}][\text{F}] = {}^{Al}K_d(\text{E-Al}) \times {}^{Al}K_a(\text{F}) \quad (4)$$

When half of the total aluminum is removed from the enzyme and assuming that no significant E-F is present, the concentrations of enzyme and E-Al are equal. Therefore, equation simplifies to

$$[\text{Al-F}]/([\text{F}]_{Total} - [\text{Al-F}]) = {}^{Al}K_d(\text{E-Al}) \times {}^{Al}K_a(\text{F}) \quad (5)$$

Half-maximal removal of aluminum (20 µM) was achieved at 29.8 µM NaF (fig. 5). We then substituted the value of ${}^{Al}K_a(F)$ [44] and of $[\text{Al-F}]/([\text{F}]_{Total} - [\text{Al-F}])$ and calculated the dissociation constant for E-Al:

$$[20.0 \times 10^{-6}]/([23.8 \times 10^{-6}] - [20.0 \times 10^{-6}]) = {}^{Al}K_d(\text{E-Al})(1 \times 10^6)$$

Therefore, ${}^{Al}K_d(\text{E-Al}) = 5.3$ µM. The K_d value of 5.3 µM is almost consistent with the K_M value of 7.7 µM obtained from the pseudo-first-order kinetic study (fig. 1B).

CD measurement of the GDH-aluminum complex

The CD spectra of GDH and the GDH-aluminum complex are shown in figure 6. At acidic pH, the spectra of the intact GDH and GDH-aluminum complex show different overall ellipticity (fig. 6A). In the far-UV region, GDH and the GDH-aluminum complex revealed a well-resolved negative peak around 208 and 222 nm. The shape of the spectra and strong negative ellipticities at 208 and 222 nm suggest that GDH is composed of α-helix- and β-sheet-rich regions. It also appears to belong to the $\alpha + \beta$ class of proteins [46]. The signal at 222 nm was greater in magnitude, which indicates the high level of structural integrity of the enzyme. The mean residue ellipticity at 222 nm was $-8.5 \pm 0.5 \times 10^3$ deg cm^2 dmol^{-1} and $-6.1 \pm 0.5 \times 10^3$ deg cm^2 dmol^{-1} for GDH and the GDH-aluminum complex, respectively. Since random and β conformations approach zero at 208 nm, the signal at 208 nm is due to an α helix conformation. This result indicates that the binding of aluminum to the enzyme induced a decrease in the ordered configuration (α helix and β-pleated sheet) and an increase in random coil. Therefore, the change in overall ellipticity at pH 6.5 may be due to the partial unfolding of the molecule induced by aluminum binding. However, for the far-UV CD spectra at pH 8.5,

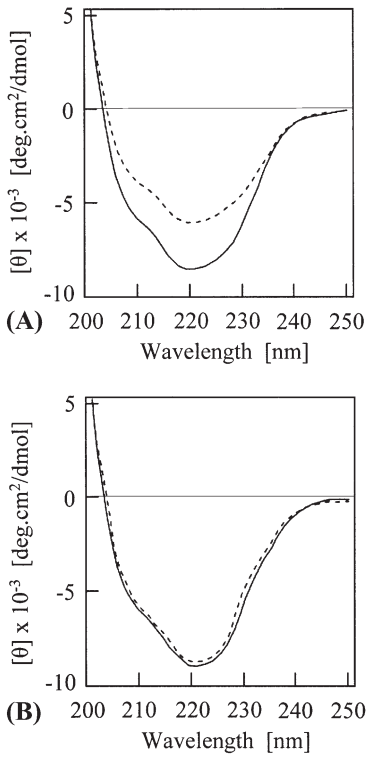

Figure 6. CD spectra. Proteins were incubated with or without 50 µM $AlCl_3$ in 25 mM HEPES, pH 6.5 (*A*) or pH 8.5 (*B*) at 25 °C for 30 min and far-UV CD spectra were monitored from 200 to 250 nm. The spectra were recorded as a five-scan average value. CD spectra are expressed in terms of [θ] (deg cm^2 $dmol^{-1}$). Solid line, GDH only; dashed line, GDH + aluminum. (*A*) CD spectra at pH 6.5. (*B*) CD spectra at pH 8.5.

all of the spectral features remain unchanged relative to the intact enzyme at 220–222 nm and 208 nm (fig. 6B).

Discussion

The results presented here establish the sensitivity of human GDH to a low level of aluminum. The sensitivity of the human GDH to aluminum suggests that inhibition of this enzyme may be relevant to aluminum toxicity. The result of the pseudo-first-order reaction of aluminum binding to GDH is very similar to that of the binding of aluminum to glucose-6-phosphate dehydrogenase [39] or binding of beryllium to phosphoglucomutase [47]. One should note that aluminum concentrations presented in the present study are those of aluminum added to the solution, and the calculated K_d value of 5.3 µM is based on the total concentration of aluminum. The inhibition is more pronounced at acidic pH and this is consistent with the high solubility of aluminum at low pH. Also noteworthy is that aluminum can modify the blood-brain barrier and become differentially compartmentalized in the various subcellular organelles in a pH-dependent manner [48]. The suggestion that aluminum mainly functions at acidic pH [49], therefore, may be a general characteristic of the reactions involving this metal ion. Solubility of aluminum salts is very high at acidic pH and very low at physiological pH. This would explain why aluminum is a better inhibitor of GDH at acidic pH. The free Al_3^+ concentration rather than the much greater $Al(OH)_4^-$ concentration will therefore be the relevant quantity in assessing ligation and in determining stability constants for such an association. In biological systems, the solubility of aluminum also varies with different concentrations of aluminum binder [50]. Therefore, the aluminum concentrations, at which inhibitory effects on enzymatic reaction occur, will very likely be much less than those observed in this study as well as in numerous studies previously reported by others [9, 31].

The protection of GDH by citrate against aluminum-induced inactivation (fig. 4) is very similar to the previous observation [51], showing that a molar ratio of 10:1 for [citrate]/[calmodulin] can prevent aluminum binding to calmodulin as determined by fluorescence and CD spectroscopy. The importance of the order of addition of the chelators to produce the observed effects (figs 3, 4) is of particular interest and similar to that observed for hexokinase [52]. HEDTA, a potential aluminum chelator [12, 53], reacted as a better chelator when it was preincubated with aluminum before formation of the enzyme-aluminum complex (figs 3, 4). A very similar protection was also reported for the aluminum binding to RNA polymerase [54] in which preincubation of RNA polymerase with 0.5 mM aluminum decreased the rate of incorporation of nucleotides by 50%, but when aluminum was premixed with the nucleotides or with the template, there was no inhibition until the aluminum concentration exceeded 2 mM.

Transferrin has been postulated as the main protein carrier of aluminum [55, 56] and the uptake of aluminum into cells appears to be largely determined by the distribution of transferrin receptors located on the cell surface [57, 58]. The transferrin receptors are present at a high density in some regions of the brain and genetic variation in transferrin has been detected in Alzheimer's disease patients [59–61]. However, the mechanism of aluminum transport from the biological fluids to the specific organs by transferrin-receptor-mediated endocytosis is not understood. Interestingly, the most recent reports have shown that no interaction is detected between transferrin receptor 1 and aluminum-saturated or mixed C site iron-loaded/N site aluminum-loaded transferrin [62]. Therefore, the fact that aluminum can be solubilized by serum transferrin in biological fluids does not necessarily imply that its transfer from the bloodstream to cytoplasm follows the receptor-mediated pathway of iron transport by transferrins. In the present study, we examined the effects of transferrin on inactivation of GDH by aluminum. The

effects of transferrin on the reactivation of the aluminum-inactivated GDH were much weaker than those of NaF or citrate (fig. 3) and complete recovery of activity was never observed even after 1 h incubation.

We also examined the effects of NGF and ferritin on inactivation of GDH by aluminum. Recently, Ohyashiki et al. [63] reported that NGF protects PC12 cells from aluminum-induced cell death. Ferritin, another iron storage protein, can bind to aluminum and aluminum causes a concentration-dependent decrease in the initial rate of iron loading into horse spleen and human brain ferritin [64, 65]. The results in figure 3 show that both NGF and ferritin have no effects on the inactivation of GDH by aluminum. However, the results for transferrin, NGF or ferritin shown in figure 3 cannot be directly transposed in vivo and do not imply that these proteins have no effects on the aluminum toxicity in a living species.

Inhibition of GDH by aluminum appears likely due to conformational changes induced by aluminum binding to carboxyl or hydroxyl groups. The result of CD analysis (fig. 6) supports this possibility. The results of CD studies are very similar to those observed for structural changes induced by aluminum in calmodulin [66], showing that the aluminum-calmodulin structure is a more random, open polypeptide than the structure of Ca-calmodulin on the basis of EPR resonance studies. Furthermore, aluminum-induced structural changes in calmodulin decrease helical content and increase random coiling and hydrophobic surface expression [67].

Environmental pollution by soluble aluminum ion has elevated aluminum from a 'harmless' to a 'toxic' metal ion. Indeed, aluminum is one of the major toxicants to plants and animals [68]. The concentration of aluminum that inactivates the human GDH in the present study is in a similar range to that observed in the plasma of uremic subjects undergoing long-term dialysis treatment [69] and in certain focal areas of the brains of patients with Alzheimer's disease [11]. Alteration of glutamate is known to be associated with neurodegenerative disorders [70]. Glutamate is a potential binder of aluminum in physiological solutions [19]. Aluminum can cross the blood-brain barrier as glutamate complex and can accelerate the aging process [20]. Aluminum is reported to potentiate glutamate-induced calcium accumulation in cerebellar granule cells [71] and enhance glutamate-mediated cytotoxicity in hippocampal cell cultures [14]. The varied responses of regional glutamate level to aluminum exposure may affect region-specific glutamate metabolism as well as activities of associated enzymes [21, 31]. Therefore, aluminum-induced alterations in enzymes of the glutamate system may be one of the causes of aluminum-induced neurotoxicity. However, a direct relationship between aluminum and glutamate toxicity as well as a unified hypothesis underlying aluminum toxicity in vivo remain to be fully demonstrated. Further investigations of the effects of aluminum are necessary for other glutamate-binding proteins for us to understand the complete pattern of glutamate metabolism disturbances by aluminum in the brain.

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1 Campbell A., Yang E. Y., Tsai-Turton M. and Bondy S. C. (2002) Pro-inflammatory effects of aluminum in human glioblastoma cells. Brain Res. **933:** 60–65
2 Ghribi O., Herman M.M., DeWitt D. A., Forbes M. S. and Savory J. (2001) Aβ(1–42) and aluminum induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of gadd 153 and NF-κB. Mol. Brain Res. **96:** 30–38
3 Alleva E., Rankin J. and Santucci D. (1998) Neurobehavioral alteration in rodents following developmental exposure to aluminum. Toxicol. Ind. Health **14:** 209–221
4 Johnson V. J. and Sharma R. P. (2003) Aluminum disrupts the pro-inflammatory cytokine/neurotrophin balance in primary brain rotation-mediated aggregate cultures: possible role in neurodegeneration. Neurotoxicology **24:** 261–268
5 Exley C., Price N. C. and Birchall J. D. (1994) Aluminum inhibition of hexokinase activity in vitro: a study in biological availability. J. Inorg. Biochem. **54:** 297–304
6 Xu Z.-X., Fox S., Melethil L., Winberg L. and Badr M. (1990) Mechanism of aluminum-induced inhibition of hepatic glycolysis: inactivation of phosphofructokinase. J. Pharmacol. Exp. Ther. **254:** 301–305
7 Kumar S. (1998) Biphasic effect of aluminum on cholinergic enzyme of rat brain. Neurosci. Lett. **248:** 121–123
8 Zatta P., Zambenedetti P. and Milanese M. (1999) Activation of monoamine oxidase type-B by aluminum in rat brain homogenate. NeuroReport **10:** 3645–3648
9 Altmann P., Al-Salihi F., Butter K., Cutler P., Blair J., Leeming R. et al. (1987) Serum aluminum levels and erythrocyte dihydropteridine reductase activity in patients on hemodialysis. N. Engl. J. Med. **317:** 80–84
10 Angeletti M., Lupidi G., Eleuteri A. M., Tacconi R., Fioretti E. and Coletta M. (1997) Effect of aluminum on the binding properties of chymotrypsin. J. Biol. Inorg. Chem. **2:** 320–326
11 Zatta P., Zambenedetti P., Pizziuti A. and Perazzolo M. (1995) Different effects of aluminum upon carbonic anhydrase and Na^+/K^+-ATPase activities in rat. Neurosci. Lett. **197:** 65–68
12 Flora S. J. S., Mehta A., Satsangi K., Kannan G. M. and Gupta M. (2003) Aluminum-induced oxidative stress in rat brain: response to combined administration of citric acid and HEDTA. Comp. Biochem. Physiol. C **134:** 319–328
13 Micic D. V., Petronijevic N. D. and Vucetic S. S. (2003) Superoxide dismutase activity in the mongolian gerbil brain after acute poisoning with aluminum. J. Alz. Dis. **5:** 49–56
14 Matyja E. (2000) Aluminum enhances glutamate-mediated neurotoxicity in organotypic cultures of rat hippocampus. Foila Neuropathol. **38:** 47–53
15 Yokel R. (2000) The toxicology of aluminum in the brain: a review. Neurotoxicology **21:** 813–828
16 Nayak P. (2002) Aluminum: impacts and disease. Environ. Res. **89:** 111–115
17 Wong P. C. L., Lai J. C. K., Lim L. and Davison A. N. (1981) Selective inhibition of L-glutamate and gamma aminobutyrate transport in nerve ending particles by aluminum, manganese, and cadmium chloride. J. Inorg. Biochem. **14:** 253–260

18 Lai J. C., Lim D. and Davison A. N. (1982) Effects of Cd^{2+}, Mn^{2+}, and Al^{3+} on rat brain synaptosomal uptake of noradrenaline and serotonin. J. Inorg. Biochem. **17:** 215–225

19 De Voto E. and Yokel R. A. (1994) The biological speciation and toxicokinetics of aluminum. Environ. Health Perspect. **102:** 940–951

20 Deloncle R., Huguet F., Fernandez B., Quellard N., Babin P. and Guillard O. (2001) Ultrastructural study of rat hippocampus after chronic administration of aluminum L-glutamate: an acceleration of the aging process. Exp. Gerontol. **36:** 231–244

21 Hofstetter J. R., Vincent I., Bugiani O., Ghetti B. and Richter J. A. (1987) Aluminum-induced decrease in choline acetyltransferase, tyrosine hydroxylase and glutamate decarboxylase in selected regions of rabbit brain. Neurochem. Pathol. **6:** 177–193

22 Nayak P. and Chatterjee A. K. (2001) Effects of aluminum exposure on brain glutamate and GABA systems: an experimental study in rats. Food Chem. Toxicol. **39:** 1285–1289

23 El-Rahman S. S. A. (2003) Neuropathology of aluminum toxicity in rats: glutamate and GABA impairment. Pharmacol. Res. **47:** 189–194

24 Hussain M. H., Zannis V. I. and Plaitakis A. (1989) Characterization of glutamate dehydrogenase isoproteins purified from the cerebellum of normal subjects and patients with degenerative neurological disorders and from human neoplastic cell lines. J. Biol. Chem. **264:** 20730–20735

25 Plaitakis A., Flessas P., Natsiou A. B. and Shashidharan P. (1993) Glutamate dehydrogenase deficiency in cerebellar degenerations: clinical, biochemical and molecular genetic aspects. Can. J. Neurol. Sci. **3:** S209–S116

26 Iwatsuji K., Nakamura S. and Kameyama M. (1989) Lymphocyte glutamate dehydrogenase activity in normal and neurological diseases. Gerontology **35:** 218–224

27 Gorovitis R., Avidan N., Avisar N., Shaked I. and Vardimon L. (1997) Glutamine synthetase protects against neuronal degeneration in injured retinal tissue. Proc. Natl. Acad. Sci. USA **94:** 7024–7029

28 Plaitakis A. and Shashidharan P. (2000) Glutamate transport and metabolism in dopaminergic neurons of substantia nigra: implications for the pathogenesis of Parkinson's disease. J. Neurol. **247:** II25–II35

29 Cavallaro S., Meiri N., Yi C. L., Musco S., Ma W., Goldberg J. et al. (1997) Late memory-related genes in the hippocampus revealed by RNA fingerprinting. Proc. Natl. Acad. Sci. USA **94:** 9669–9673

30 Dutuit M., Didier-Bazes M., Vergnes M., Mutin M., Conjard A., Akaoka H. et al. (2000) Specific alteration in the expression of glial fibrillary acidic protein, glutamate dehydrogenase, and glutamine synthetase in rats with genetic absence epilepsy. Glia **32:** 15–24

31 Zatta P., Lain E. and Cagnolini C. (2000) Effects of aluminum on activity of Krebs cycle enzymes and glutamate dehydrogenase in rat brain homogenate. Eur. J. Biochem. **267:** 3049–3055

32 Yang X., Bi S., Wang X., Liu J. and Bai Z. (2003) Multimethod characterization of the interaction of aluminum ion with alpha-ketoglutaric acid in acidic aqueous solutions. Anal. Sci. **19:** 273–279

33 Cho S.-W., Yoon H.-Y., Ahn J.-Y., Lee E.-Y. and Lee J. (2001) Cassette mutagenesis of lysine 130 of human glutamate dehydrogenase: an essential residue in catalysis. Eur. J. Biochem. **268:** 3205–3213

34 Lee E. Y., Yoon H. Y., Ahn J. Y., Choi S. Y. and Cho S.-W. (2001) Identification of GTP binding site of human glutamate dehydrogenase using cassette mutagenesis and photoaffinity labeling. J. Biol. Chem. **276:** 47930–47936

35 Yoon H.-Y., Lee E.-Y. and Cho S.-W. (2002a) Cassette mutagenesis and photoaffinity labeling of adenine binding domain of ADP regulatory site within human glutamate dehydrogenase. Biochemistry **41:** 6817–6823

36 Yoon H.-Y., Cho E. H., Kwon H. Y., Choi S. Y. and Cho S.-W. (2002b) Importance of glutamate-279 for the coenzyme binding of human glutamate dehydrogenase. J. Biol. Chem. **277:** 41448–41454

37 D'Haese P. C., Van de Vyver F. L., Wolff F. A. de and DeBroe M. E. (1985) Measurement of aluminum in serum, blood, urine, and tissues of chronic hemodialyzed patients by use of electrothermal atomic absorption spectrometer. Clin. Chem. **31:** 24–29

38 Cho S.-W., Lee J. and Choi S. Y. (1995) Two soluble forms of glutamate dehydrogenase isoproteins from bovine brain. Eur. J. Biochem. **233:** 340–346

39 Cho S.-W. and Joshi J. G. (1989) Time-dependent inactivation of glucose-6-phosphate dehydrogenase from yeast by aluminum. Toxicol. Lett. **47:** 215–219

40 Yang J. T., Wu C.-S. C. and Martinez H. M. (1986) Calculation of protein conformation from circular dichroism. Methods Enzymol. **130:** 208–269

41 Balasubramanian D. and Kumar C. (1976) Recent studies of the circular dichroism and optical rotatory dispersion of biopolymers. Appl. Spectrosc. Rev. **11:** 223–286

42 Karlik S. J. and Eichhorn G. L. (1989) Polynucleotide cross-linking by aluminum. J. Inorg. Biochem. **37:** 259–269

43 Smith T. J., Schmidt T., Fang J., Wu J., Siuzdak G. and Stanley C. A. (2002) The structure of apo human glutamate dehydrogenase details subunit communication and allostery. J. Mol. Biol. **318:** 765–777

44 Brosset C. and Orring J. (1943) Studies on the consecutive formation of aluminum fluoride complexes. Sven. Kem. Tidskr. **55:** 101–117

45 Lange A. J., Arison W. J., Burchell. A. and Burchell B. (1986) Aluminum ions are required for stabilization and inhibition of hepatic microsomal glutamate-6-phosphatase by sodium fluoride. J. Biol. Chem. **261:** 101–107

46 Manavalan P. and Johnson W. C. (1983) Sensitivity of circular dichroism to protein tertiary structure class. Nature **305:** 831–832

47 Hashimoto T., Joshi J. G., Rio C. D. and Handler P. (1967) Phosphoglucomutase. J. Biol. Chem. **242:** 1671–1679

48 Banks W. A., Kastin A. J. and Zatta P. (1995) The blood-brain aluminum toxicity and Alzheimer's disease. In: Non Neuronal Cells in Alzheimer's Disease, pp. 1–12, Zatta P. and Nicolini M. (eds), Word Scientific, Singapore

49 MacDonald T. L. and Martin R. B. (1988) Aluminum ion in biological systems. Trends Biochem. Sci. **13:** 15–19

50 Martin R. B. (1986) The chemistry of aluminum as related to biology and medicine. Clin. Chem. **32:** 1797–1806

51 Suhayda C. G. and Haug A. (1984) Organic acids prevent aluminum-induced conformational changes in calmodulin. Biochem. Biophys. Res. Commun. **119:** 376–381

52 Womack F. C. and Colowick S. P. (1979) Proton-dependent inhibition of yeast and brain hexokinases by aluminum in ATP preparations. Proc. Natl. Acad. Sci. USA **76:** 5080–5084

53 Graff L., Muller G. and Burnel D. (1995) In vitro and in vivo comparative studies on chelation of aluminum by some polyaminocarboxylic acids. Res. Commun. Mol. Pathol. Pharmacol. **88:** 271–292

54 Crapper-McLanchlan D. R., Farnell B., Galin H., Karlik S., Eichhorn G. and De Boni U. (1983) Biological Aspects of Metal and Metal-Related Disease, pp. 209–218, Raven, New York

55 Cochran M., Chawtur V., Jones M. E. and Marshall E. A. (1991) Iron uptake by human reticulocytes at physiologic and subphysiologic concentrations of iron transferrin: the effect of interaction with aluminum transferrin. Blood **77:** 2347–2353

56 Ward R. J., Zhang Y. and Crichton R. R. (2001) Aluminum toxicity and iron homeostasis. J. Inorg. Biochem. **87:** 9–14

57 McGregor S. J., Naves M. L., Oria R., Vass J. K. and Brock J. H. (1990) Effect of aluminum on iron uptake and transferrin-re-

ceptor expression by human erythroleukaemia K562 cells. Biochem. J. **272:** 377–382
58 Roskams A. J. and Connor J. R. (1990) Aluminum access to the brain: a role for transferrin and its receptor. Proc. Natl. Acad. Sci. USA **87:** 9024–9027
59 Edwardson J. A., Ferrier I. N., McArthur F. K., McKeith I. G., McLaughin I., Morris C. M. et al. (1991) Alzheimer's disease and the aluminum hypothesis. In: Aluminum in Chemistry, Biology, and Medicine, pp. 84–96, Nicolini M., Zatta P. F. and Corain B. (eds), Raven, New York
60 Van Rensburg S. J., Carsten M. E., Potocnick F. C., Aucamp A. K. and Taljaard J. J. (1993) Increased frequency of the transferrin C2 subtype in Alzheimer's disease. NeuroReport **4:** 1269–1271
61 Namekata K., Imagawa M., Terashi A., Ohta S., Oyama F. and Ihara Y. (1997) Association of transferrin C2 allele with late-onset Alzheimer's disease. Hum. Genet. **101:** 126–129
62 Hemadi M., Miquel G., Kahn P. H. and Chahine J.-M. E. H. (2003) Aluminum exchange between citrate and human serum transferrin and interaction with transferrin receptor 1. Biochemistry **42:** 3120–3130
63 Ohyashiki T., Satoh E., Okada M., Takadera T. and Sahara M. (2002) Nerve growth factor protects against aluminum-mediated cell death. Toxicology **176:** 195–207
64 Fleming J. and Joshi J. G. (1991) Ferritin: the role of aluminum in ferritin function. Neurobiol. Aging **12:** 413–418
65 Dedman D. J., Treffry A. and Harrison P. M. (1992) Interaction of aluminum citrate with horse spleen ferritin. Biochem. J. **287:** 515–520
66 Siegel N., Coughlin R. and Haug A. (1983) A thermodynamic and electron paramagnetic resonance study of structural changes in calmodulin induced by aluminum binding. Biochem. Biophys. Res. Commun. **115:** 512–517
67 Siegel N. and Haug A. (1983) Aluminum interaction with calmodulin: evidence for altered structure and function from optical and enzymatic studies. Biochim. Biophys. Acta **744:** 36–45
68 Savory J., Exley Ch., Forbes W. F., Huang Y., Joshi J. G., Kruck T. et al. (1996) Can the controversy of the role of aluminum in Alzheimer's disease be resolved? What are the suggested approaches to this controversy and methodological issues to be considered? J. Toxicol. Environ. Health **48:** 615–635
69 Alfrey A. C. (1991) Aluminum intoxication recognition and treatment. In: Aluminum in Chemistry, Biology, and Medicine, pp. 73–84, Nicolini M., Zatta P. F. and Corain B. (eds), Raven, New York
70 Choi D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron **1:** 623–634
71 Mundy W. R., Freudenrich T. and Kodavanti P. R. (1997) Aluminum potentiates glutamate-induced calcium accumulation and iron-induced oxygen free radical formation in primary neuronal cultures. Mol. Chem. Neuropathol. **32:** 41–57