To the Graduate Council:

I am submitting herewith a dissertation written by James Thomas Fleming entitled "Studies on Brain Ferritin." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

J.G. Joshi, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Provost and
Dean of the Graduate School

STUDIES ON BRAIN FERRITIN

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

James Thomas Fleming

May 1989

Dedication

To Mickey

Without whose support

I would have been unable to continue

ACKNOWLEDGMENTS

I would like to thank Dr. Jayant G. Joshi for his constant inspirational and financial support. His emphasis on scientific rigor and the primacy of publishing will insure success in my career as a biochemist. I would also like to thank my committee members Dr. Jorge E. Churchich, Dr. Dan M. Roberts and Dr. Walter R. Farkas for taking a personal interest in my development. Let me also thank my colleagues, Dr. Steven Sczekan, Dr. Sung-woo Cho and Martin Clauberg all of whom taught me a great deal both about biochemistry and friendship. Last and certainly not least, I would like to thank my parents for their love and encouragement.

ABSTRACT

Ferritin was isolated from the livers and brains of two groups of rats, one of which was fed $AlCl_3$ (100μM) for one year in the drinking water. Brain contained about a third of the ferritin found in the liver. While brain ferritin from normal rats contained 42.1 ± 14.3 g atoms of aluminum (Al^{+3}), that from the Al^{+3}-fed group contained 115.4 ± 48.3 g atoms per mole of ferritin. Liver ferritin from both groups contained less Al^{+3} and and more iron (Fe) compared with brain ferritin.

Ferritin isolated from the brains of patients who died of Alzheimer's disease (AD) contained more Al^{+3} and more iron than that from age-matched controls. Human brain ferritin (HBF) is composed of two types of subunits, about 70% heavy (Mr 22,000) and 30% light (Mr 19,500). The isoelectric focusing pattern of HBF was considerably different from that of human liver ferritin (HLF). Only five of the twenty brain ferritin bands migrated similarly to the acidic isoferritins from the liver and the major component of human brain ferritin, representing 30% of the total ferritin, had a pI of 8.

We have further characterized rat brain ferritin and human brain ferritin isolated form both AD and normal brain tissue with respect to the effect of Al^{+3} on iron loading and release and subunit composition. Al^{+3} causes a

concentration dependent decrease in the initial rate of iron loading into demetallo-apo human brain ferritin and the rates were similar for ferritin isolated from both AD and normal tissue. The rates of iron release were: horsespleen ferritin (HSF) >> HLF > RBF > HBF = rat liver ferritin. The rates of iron release from AD and Normal ferritin were similar and were unaffected by preloading with Al^{+3}. Several mammalian ferritins were compared for their total iron uptake; HSF > HLF > HBF. Comparison of human brain ferritin from AD and N brain tissue showed no statistical difference in their iron uptake. Consistent with our earlier observations, holoferritin is more resistant to precipitation by Al^{+3} than apoferritin. Human brain ferritin displays two subunits on SDS-PAGE: Mr 22,000(H) and 19,000(L) which show immunological reactivity with anti-human H chain and anti-human liver ferritin (L) antibodies, respectively. Rat brain ferritin displays three subunits on SDS-PAGE Mr 23,000(H), 22,000(M) and 20,000(L). Only the L chain reacted with anti-human L chain antibodies. The H and M chains did not react with anti-human H chain or anti-human L chain antibodies.

Concavalin A (Con A) is reported to be a ferritin. However this protein had no amino acid sequence homology with any of the known ferritins and did not react specifically with either anti-human brain or anti-soybean

ferritin antibodies.

Reverse phase HPLC resolved human brain ferritin into clusters of H (7 peaks) and L(5 peaks) components. The two major H peaks represented 24% and 41% of the total H and one major L peak represented 74% of the total L protein. The cluster of H-chain peaks migrated with an R_f on SDS-PAGE similar to that of the human brain ferritin H chain. Amino acid analysis of two homogeneous H chain peaks were very similar. Comparison of the amino acid composition of human brain ferritin H and L chains with the respective human liver ferritin subunits shows that while they are similar, the human brain ferritin H chain has fewer lys residues and the human brain ferritin L chain has more glx residues. Human brain homogenates in sucrose were fractionated by centrifugation. The ferritin distribution was: cellular debris 19.3%, nuclear 1.7%, mitochondrial 5.4%, microsomal 15.6% and cytosolic 58.0%. Most of the iron was found in the cellular debris (60%) and microsomal fractions (26%). While serum ferritin is a glycoprotein, SDS-PAGE gels of human brain and rat brain ferritin did not stain for carbohydrates. These data suggest that the L chain is more conserved between species and H chain heterogeneity implicates the H chain in differing isoferritin functions.

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INTRODUCTION

Aluminum (Al^{+3}), the most abundant metal in the Earth's crust, has until recently been found in very low concentrations in ground and surface waters because of its relative insolubility at neutral pH (1). With the advent of industrialization came an increase in acid precipitation which in turn has caused a loss of exchangable ions in the soil and the release of normally sparingly soluble Al^{+3} (1). Organisms that evolved in a neutral pH environment have, therefore, only recently had to cope with appreciable Al^{+3} water levels (2). Al^{+3} has long been known to impair growth of crop plants and is a factor in forest decline in North America and Europe (3). While once the acidity of fresh water lakes was thought to be directly responsible for the death of aquatic populations, present opinion now puts the blame on Al^{+3} (4). The most extensive research on Al^{+3} toxicity has focused on its involvement in several human central nervous system disorders including dialysis encephalopathy, amylotrophic lateral sclerosis, and Alzheimer's disease.

Patients with chronic renal malfunction that undergo dialysis effectively concentrate Al^{+3} from trace levels in the dialysis fluid (5). In addition, the majority of patients ingest large amounts of phosphate (PO_4) binding Al^{+3} gels to decrease phosphate absorption

and prevent hyperphosphatemia. The combined effect of this exposure is to concentrate significant amounts of Al^{+3} in various tissues including bone and brain. Dialysis encephalopathy is characterized by language disorders, seizures, myoclonus, dyspraxia, and dementia (5). Brain Al^{+3} content is substantially elevated in patients dying with this disorder (6).

In southern Guam, Kii peninsula in Japan and western New Guinea, where soils are high in Al^{+3} and low in magnesium (Mg^{+2}) and calcium (Ca^{+2}) the population suffers from a high frequency of amyotrophic lateral sclerosis, a form of Parkinson's disease with severe dementia. The onset of the disease usually occurs at a late age and the neuropathology is similar to that of age-related changes. Histopathological studies show accumulation of Al^{+3} and Ca^{+2} in degenerated brain areas and neurofibrillary degeneration (7).

Alzheimer's disease (AD) is a progressive dementia in which the pathological findings are atrophy of the brain, loss of some neurons, decrease in the arborization of the dendritic tree, neurofibrillary tangles, neuritic plaques and amyloid plaques (8). The number of neurofibrillary tangles and neuritic plaques appears to correlate with the degree of dementia (9). Neurofibrillary tangles, the most striking feature of the AD brain, are made of a pair of

helically wound 10 nm filaments with a twist every 80 nm (8).

Neurochemical studies by Crapper et al. of brain tissue from patients with AD found elevated total brain Al^{+3} levels which led investigators to believe Al^{+3} may play a role in the etiology of the disease (10). Since this first report eight independent labs around the world have reported finding elevated brain Al^{+3} levels in AD patients (11). In addition, a number of studies have reported Al^{+3} colocalized with neurofibrillary tangles and neuritic plaques. Perl, using a combination of electron microscopy and X-ray spectroscopy, detected foci of Al^{+3} within the nuclear region of a high percentage of hippocampal neurons containing neurofibrillary tangles from the cases of senile dementia as well as elderly controls (12). In contrast, the adjacent normal appearing neurons in both groups of patients were free of detectable Al^{+3}. Candy et al., using energy dispersive X-ray microanalysis and high resolution solid-state nuclear magnetic resonance found the co-localization of Al^{+3} and silicon in senile plaque cores isolated from the cerebral cortex of patients with AD (13).

Epidemiological evidence of a relation between Al^{+3} and AD has been difficult to obtain for a number of reasons. The reliability of reported water Al^{+3} levels is frequently suspect because of the difficulty in analysis

and possible contamination of samples (14). Histological confirmation of neurofibrillary tangles is the currently accepted criteria for diagnosis of AD and there is a lack of an accepted behavioral or non-invasive diagnostic criteria that would be required for a statistically significant epidemiological study. Recently, however, several researchers have demonstrated that in about 80% of cases, a diagnosis made on clinical features alone is in in aggrement with the neuropathological findings (15,16). Computer Tomographic (CT) scanning has also been shown useful in the diagnosis of AD (17). In a recent study to examine the possible relation between exposure to Al^{+3} in water and the development of AD, a CT scanning survey of 4,100 people in eighty-eight county districts within Wales and England was conducted (18). The risk of contracting AD was found to be 1.5 times higher in the districts where the mean Al^{+3} concentration exceeded 0.11 mg/l than in districts where concentrations were less that 0.11 mg/l. While this is the best evidence to date linking Al^{+3} water levels and AD, it is not sufficient to establish a causal connection (18).

While a general pattern relating Al^{+3} and dementia is suggested, several inconsistencies arise in the attempt to relate the above mentioned neurological findings into a general Al^{+3} neurotoxicity model. Al^{+3} has been shown to

induce neurofibrillary changes in several species of animals by intercranial injection but they are composed of 10nm filaments and not of paired helical filaments as seen in AD brain tissue (19). In Al^{+3} treated animals the spinal cord neurons also show neurofibrillary changes while in AD no spinal neural changes are seen (20). The neuritic plaques associated with neurofibrillary tangles in AD are also not seen in Al^{+3} treated animals (20). Cerebral transplants from aborted human fetuses cultured in vitro and exposed to Al^{+3} develop 10 nm filaments similar to those produced in experimental animals but unlike those seen in AD (21). Patients with renal dialysis encephalopathy have high levels of total brain Al^{+3} but do not show AD like pathology (6) Also related to the Al^{+3}-neurotoxicity model is the question of whether Al^{+3} causes the primary etiology in AD or secondarily has affinity for the damaged neural tissue.

Despite these inconsistencies, the presence of Al^{+3} as found in neurofibrillary tangles and neuritic plaques raises questions as to the mode of absorption of Al^{+3} in the gut and transport to the central nervous system. The intake of Al^{+3} is estimated to be about 20 mg/ day (range 1-100) via the gastrointestinal tract and 3-15 μg/day (range 2-100) via inhalation, with plasma levels of 7 μg/l (range 1.5-15) (22). Because the ionic radius and

hydrolysis behavior of Al^{+3} and iron are similar, it is reasonable to assume that Al^{+3} is transported in the extracellular and intracellular environments by the same mechanism that transports iron (22). This position is supported by studies that have shown Al^{+3} interacts with physiological Fe ligands such as transferrin (23), ferritin (24), lactoferrin (23), citrate (25), and phosphate (25).

Transferrin stands as the leading plasma protein for Al^{+3} binding. Transferrin receptors have been found on brain capillaries and bidirectional transport of transferrin across the blood brain barrier has been demonstrated (26,27). At a plasma concentration of 3 mg/ml, with a 30% occupancy by iron transferrin provides 50 μM metal binding sites (1). In addition, with citrate-to-transferrin molar ratio comparable to that found in the plasma, citrate releases both Al^{+3} and Fe to transferrin (1). Stability of the Al^{+3}-transferrin complex is believed to be less than that of ferric iron, but it is still high enough to suppress the interaction of Al^{+3} with the previously mentioned plasma ligands (1). Therefore Al^{+3} and Fe most probably gain entry into the cell by the same transferrin mediated process (22). The extremely low Al^{+3} flux into cells and across the blood brain barrier may explain why the brain may be specifically affected; short lived cells, unlike neurons, are not able to

accumulate toxic levels of Al^{+3} (22).

Ferritin is an oligomeric protein of Mr 450,000, composed of at least two types of subunits with mol. wts. of 19,000 (L) and 21,000 (H). The subunits form a protein shell which can sequester up to 4500 mol of Fe^{+3} as ferric hydroxyphosphate per mole of ferritin (28). Fe^{+2} in the presence of dioxygen can catalyze the production of active oxygen species (29) leading to the propagation of free radicals which are implicated in lipid peroxidation (30). Ferritin thus allows storage of iron in a non-toxic Fe^{+3} state, the protein shell, in effect, affording solubility to the otherwise insoluble Fe^{+3} core. While the mechanism of in vivo Fe^{+2} release from ferritin is not known, the process may be modeled in vitro using reductants such as $FMNH_2$ or ascorbate. The converse process, Fe^{+3} loading may also be modeled in vitro using an oxidizing buffer and Fe^{+2} (31).

Ferritin synthesis is induced by iron and is controlled at both the transcriptional and translational levels. Changes in mRNA levels have been observed in cells for which iron is used intracellularly during development, differentiation and iron overload (32). In cells that store iron for other cells, such as hepatocytes, the translation of ferritin mRNA is induced by an increase in cytoplasmic iron levels. This translational mechanism ensures rapid

sequestering of iron thereby preventing any toxic effects of the metal (33). The critical regulatory region responsible for this Fe-dependent translational regulation has been found within the 5′-untranslated region of the human ferritin H-chain and the rat ferritin L-chain (34). Whether the transcriptional or translational mechanisms are inducible by other non-ferrous metals has yet to be determined.

Ferritin iso-forms from different tissues contain different proportions of the H and L chains, the ratios of which are determined by the iron state of the cell. There appear to be both short and long term iron loading effects on isoferritin populations. Treffry et al. (35) and Bomford et al.(36) reported an increase in rat liver L-subunit rich basic isoferritins 2 hrs. after iron injection. Bomford et al. found a predominance of acidic isoferritins in patients with idiopathic haemochromatosis. They suggested that this might be due to preferential synthesis or retarted degradation of acidic isoferritins in iron loaded states. Hoy et al. have reported an increase in acidic isoferritins 24 hrs after iron loading Chang cells (37). In addition shift in the pI spectrum, there was also an increase in the proportion of ferritin reacting to heart antibody at a given pI. Their work suggests that changes in immunoreactivity induced by iron do not depend entirely on

subunit composition and may therefore involve some post-translational modification. Acidic isoferritins are also implicated in the control of hemopoiesis of stem cells in the bone marrow (38). White et al. reported that iron caused a rapid increase in transcription of the L subunit gene, followed by a rise in L chain mRNA levels, whereas the H subunit gene transcription did not increase significantly (39). Despite the complexity of the regulation of the H and L subunits, it seems clear that different isoferritins have different functions in the cell presumably on the basis of their differing structure.

In addition to binding iron, ferritin also binds several non-ferrous metal ions including Cu^{+2}, Zn^{+2}, V^{+2}, Tb^{+3}, Cd^{+2}, Be^{+2} and Al^{+3} (40-44). Apoferritin binds approximately 2 mol metal ion/ mol ferritin subunit, except Be^{+2} which is bound at 60 mol/ mol ferritin. Holoferritin binds substantially greater amounts of these metals, e.g. up to 1000 mol Be^{+2}/mol ferritin. Ferritin may therefore play a physiological role in the detoxification of metals similar to that proposed for metallothionein (45) by effectively removing metal ions from the cellular environment.

Ferritin has been shown, <u>in vitro</u>, to protect against and reverse the Be^{+2} inactivation of Na^{+} K^{+} ATPase, alkaline phosphatase, RNA polymerase and phosphoglucomutase

(46). In addition, rats whose ferritin synthesis was elevated 5 fold by Fe^{+3} administration survived otherwise toxic doses of Be^{+2} (44). Consistent with the observed in vitro binding of non-ferrous metals to ferritin, rats injected with Zn^{+2}, Cd^{+2}, Pb^{+2}, and Be^{+2}, had a portion of the injected metal ions bound to liver ferritin (44). The values varied from 2 mol Pb^{+2}/mol ferritin to 85 mol Zn^{+2}/mol ferritin.

As evidenced by the increase in metal binding of holoferritin, the Fe core, as opposed to the protein shell has been implicated as the major site of non-ferrous metal binding. When $^{7}Be^{+2}$ loaded holoferritin was subjected to sucrose density centrifugation and subsequently assayed for Be^{+2}, Fe and phosphate (PO_4), the ratios of Be^{+2}/Fe, PO_4/Be^{+2}, and Be^{+2}/PO_4 were constant, showing colocalization of Be^{+2}, Fe,and PO_4 (47). To confirm the hypothesis that the core is primarily responsible for metal binding, the binding of several non-ferrous metals to synthetic Fe cores with and without PO_4 was determined (48). Cd^{+2}, Zn^{+2}, Be^{+2}, and Al^{+3} were bound to the synthetic cores with K_d values similar to those found for holoferritin, while the number of metal ions bound was inversely proportional to the ionic radius ($Be^{+2} > Al^{+3} > Zn^{+2} < Cd^{+2}$). The addition of PO_4 to the synthetic

cores increased the amount of bound metal without changing the Kd.

The finding of significant amounts of nonferrous metals bound to ferritin as isolated raises the question of whether or not the metals ions are bound adventitiously during isolation (32). In vivo evidence for non-ferrous metal binding has come from X-ray electron microscopy of sections of germinated soybeans that show co-localization of Al^{+3} with the crystalline arrays of ferritin in amyloplasts (49).

In addition to the detoxification of metals by effectively removing them from the cellular environment, the storage of these metals in the ferritin core may provide an ever present source of these metals which may or may not be to the cell's advantage. Zn^{+2} or Cu^{+2} requiring apoenzymes were reactivated by incubating them with ferritin preloaded with the respective metal ions suggesting that ferritin may function as a metal donor (42). Al^{+3} has been demonstrated in vitro and in vivo to inhibit a number of enzymes including acetylcholine esterase (50), glucose-6-phosphate dehydrogenase (51), GTPase (52), ferroxidase (53), catechol-O-methyl transferase (53) and the Mg^{+3} requiring enzymes, hexokinase (54), 3',5'-cyclic nucleotide phosphodiesterase (53) and alkaline phosphatase (53). In fact, the substitution of

Al^{+3} for Mg^{+2} at critical regulatory enzymes has been suggested to be the primary mechanism for Al^{+3} mediated toxicity (2). Ferritin may donate Al^{+3} to these and other enzymes, thus altering the cellular physiology. Functioning as both a metal scavenger and metal donor, brain ferritin may play a pivotal role in neural metal transport and metabolism.

In this report we describe the isolation, characterization and metal binding properties of ferritin from rat and human brain. The dissertation is presented in two parts:

[1] Ferritin: Isolation of Aluminum-Ferritin Complex from Brain.

[2] Brain Ferritin: Characterization and Role of Al^{+3} in Ferritin Function.

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CHAPTER I

FERRITIN: ISOLATION OF ALUMINUM - FERRITIN COMPLEX FROM BRAIN

INTRODUCTION

Ferritin is a ubiquitous iron storage protein with a molecular weight of about 480,000. It is composed of two types of subunits, heavy (H, Mr 22,000) and light (L, Mr. 19,500) (1). Ferritins from heart and liver contain predominantly H or L chains respectively. Synthesis of ferritin is enhanced by iron (2). A fully saturated ferritin contains up to 4500 g atoms of iron sequestered as ferrichydroxyphosphate in its protein shell (1). Since 1982 the data obtained by us and by other laboratories suggest that ferritin is a multifunctional molecule and as such (a) it detoxifies, stores and transports Fe, (b) it binds other metal ions both in vivo and in vitro (3,8) and functions as a zinc (Zn^{+2}) detoxicant and a Zn^{+} ion donor, (4) (c) in the presence of a reductant, it releases Fe^{+2} which can facilitate the generation of free radicals, (5,6) (d) it functions as a dephosphorylating agent and therefore may affect several metabolic processes (5,7). We found that holoferritin can bind unusually large quantities of beryllium (Be^{+2}) and that rats pretreated

with Fe injections resulting in elevated levels of ferritin survived otherwise toxic doses of intravenous Be^{+2} (8). An expanded role for ferritin in metal toxicity was thus suggested (7,8,9).

Although the chemical properties of Be^{+2} and aluminum (Al^{+3}) are similar (10), Be^{+2} is extremely toxic (11) but Al^{+3}, one of the most abundant elements in the earth's crust and in the environment, has until recently been considered harmless. The reported increased Al^{+3} levels in the brains of patients with dialysis dementia (12) and Alzheimer's disease (AD) (13) have led to the suggestion that Al^{+3} may be an etiological agent for dementia. Other current theories to explain the pathogenesis of AD include decreased cholinergic innervation, defective protein synthesis and a slow infectious process (14). None are unequivocally proven or ruled out.

Humans ingest about 30 mg of Al^{+3} daily, mostly as a food contaminant but only about 10 to 20 ug is actually absorbed (15). A substantial amount of the absorbed Al^{+3} is deposited in the bones and brain (15). Thus under normal physiological conditions the accumulation of Al^{+3} results from chronic low level exposure. The isolation of ferritin complexed with beryllium, zinc, copper, or cadmium from the livers of animals injected interperitoneally with

the corresponding metal salts (8) and the similarities between the inorganic properties of Be^{+2} and Al^{+3} suggested that Al-ferritin complexes might be obtained from the brains of animals which were fed even low levels of Al^{+3}. Therefore, we initiated a series of experiments to study the long-term effects of low levels of dietary Al^{+3} on the ferritin in the brain and liver. In this report we show that (i) normally, a significant amount of Al^{+3} in the brain is complexed with ferritin (ii) the ferritin isolated from the brains of Al^{+3}-fed animals has more Al^{+3} bound to it than that isolated from control animals (iii) the ferritin isolated from the brains of AD patients has more Al^{+3} bound to it than that isolated from the age-matched patients without AD and (iv) compared to liver ferritin, the isoelectrophoretic pattern of brain ferritin displays greater microheterogenity and contains less iron.

MATERIALS AND METHODS

The water used was passed through two ion exchangers and had a resistance of greater than 20 M ohms. Ultrapure HNO_3 was obtained from Baker. Metal standards used for atomic absorption spectrophotometry were obtained from Spex Industries. All gel components were obtained from Pharmacia. All other chemicals were of analytical grade.

All glassware and plasticware was rinsed with 2% HNO_3 and deionized water before use.

To determine realistic levels of Al^{+3} to be used in the animal experiments we measured the Al^{+3} content of six common beverages in bottles and cans randomly collected from local grocery stores. The pH of each sample was determined, 1ml aliquots of each were degassed under a vacuum, adjusted to 2% HNO_3 and analyzed for Al^{+3}. The amount of Al^{+3} was determined from solutions adjusted to 2% HNO_3 with an IL- 550 atomic absorption spectrometer equipped with a graphite furnace.

Twenty-two male 10-12 week old Sprague-Dawley rats obtained from Charles River breeders were divided into two groups and fed ad lib Purina Lab-lock chow (#5001) and tap water. The solid food was prepared for Al^{+3} analysis by digestion in 50% HNO_3 for 12 hrs. at 70° and subsequently diluted to 2% HNO_3. The tap water was prepared for analysis by adding HNO_3 to 2%. The Al^{+3} content of the chow and water was 8.3 mg/g dry wt. and 2.7μ respectively. The drinking water of the experimental group was supplimented with 100 μ $AlCl_3$. This was about three times the Al^{+3} concentration measured in the commercial beverages. After one year the animals were tested in a standard T-maze for learning and recall (16), sacrificed,

their brains and livers dissected, rinsed with ice cold saline and stored until used at -80° in 0.1M Tris pH 7.4.

Ferritin was isolated from the homogenates of two brains (2g/brain) and from individual livers as described previously (3) up to the point of precipitating with $(NH_4)_2SO_4$. The 40-50% saturated $(NH_4)_2SO_4$ precipitate was dissolved in a minimum volume of 5mM Na_2HPO_4 buffer pH 7.0, applied to a 1.8 x 75 cm sephacryl S-300 column equilibrated with the same buffer, and 3ml fractions were collected with UV monitoring and assayed for Fe. The Fe containing fractions were pooled, concentrated with an Amicon concentrator fitted with a PM-30 filter and stored at 5° with 0.05% sodium azide. The isolated protein was shown to be ferritin by immunoprecipitation against polyclonal rabbit antibodies against the heavy chain of human liver ferritin.

The brains from four elderly patients, two of which displayed the histological features of AD as determined by light microscopy were obtained through the Cole Neuroscience Foundation. Their age, sex and cause of death were as follows: 1) 78 years, female, small bowel volvulus with jejunal infarction and AD, 2) 80 years, female, respiratory failure due to bilateral acute necrotizing bronchopneumonia and AD, 3) 78 years, female, septic shock

secondary to urinary tract infection, 4) 55 years, male, myocardial infarction. Upon autopsy, the brains were removed, put in plastic bags and frozen at -80°. The whole brains were slightly thawed, cut into pieces and homogenized in a blender for 1 min in 0.1 M Tris-HCl pH 7.4. Ferritin was isolated using the same procedure outlined for rat brains.

Native polyacrylamide gel electrophoresis was performed using a 5% gel- barbituric acid-Tris system (17) and SDS-PAGE was performed with a 10-20% gradient gel using the ammediol system of Wychoff (18). The amount of protein isolated was determined by the method of Lowry using the appropriate correction factors (3).

Isoelectric focusing (IEF) was carried out at 10° on a Pharmacia FBE-3000 flat bed system, using a Pharmacia Ephortec 3000v power supply. Initial attempts at IEF of brain ferritin using the standard techniques (Pharmacia handbook "Isoelectric Focusing") such as 5% acrylamide or 1% agarose gels under high voltage and constant power conditions gave poor results. Electrophoresis for extended periods of time at low voltage, as described below, was essential to minimize smearing and obtain highly resolved brain ferritin bands. Therefore the most satisfactory and reproducible procedure which resulted after several trials

is described here in detail. Gels, 0.75mm thick were cast between glass plates,the inner face of one having been coated with dimethylsilane. The gel composition was 3% acrylamide, 0.09% BIS, 6.3% 3-10 pH ampholyte, 25% glycerol, 0.04% TEMED and 0.02% ammonium persulfate. 2.5 x 5.0 mm electrode strips were placed 14 cm apart and 0.1 N NaOH and 1 M acetic acid were used for the catholyte and anolyte respectively. The gel was prefocused for 3 hrs at 10^0 during which time the voltage was gradually increased from 30 to 200 v. Filter paper strips were soaked (0.5 x 1.0cm) in the appropriate ferritin solution and applied with forceps in duplicate to the gel 2 cm from the cathode. The gel was run at 100v for 5 hrs. after which the filter paper strips were removed and the gel was allowed to run overnight (14 hrs) at 10^0. The next day the voltage was gradually increased over a 5 hr. period to 2000 v, after which electrophoresis was stopped. Focusing time was determined as the time required for a sample placed at both the anode and cathode to migrate to the same pI region. The gel was placed in a pan over a thin layer of dry ice, five millimeter wide slices from one lane were placed individually into 1.5 ml centrifuge tubes, 0.5 ml of H_2O was added to each tube and left overnight. The next day the pH of each sample was determined and subsequently

concentrated HNO_3 was added to bring each tube to 2% HNO_3. The tubes were again left overnight and the iron and aluminum content of each sample was determined. The rest of the gel was fixed in 5% sulphosalicylic acid and 10% trichloroacetic acid for 1 hr, soaked in methanol: acetic acid:water (3:1:6), stained with coomassie blue, destained, dried between sheets of dialysis cellophane and scanned on a Hoefer densitometer.

Equilibrium dialysis was performed with 1 mg of ferritin from human brain, human liver, and horse spleen in 1 ml of 5 mM HEPES buffer pH 6.5. These samples were dialysed at room temperature three times for 12 hrs. each against 2 liters of the same buffer containing 20 μM $AlCl_3$. The unbound Al^{+3} was removed by further dialysis against the same buffer without Al^{+3}. A dialysis bag containing aluminum free buffer was processed simultaneously to serve as a blank. The contents of the bags were centrifuged and the supernatant was then analysed for protein, Fe and Al^{+3} by atomic absorption and for phosphate by colorimetry (19). Ferritin will precipitate at concentrations above 0.2 mM $AlCl_3$ (data not shown), but no precipitation was observed at 20 μM $AlCl_3$.

Statistical analysis was performed using a Hewlett-Packard HP-41 T-statistics program.

RESULTS AND DISCUSSION

Selecting a "low" exposure-level of Al^{+3} is difficult because of its ubiquitous presence in the environment. Common sources include dust, smoke, medications and water. Al^{+3} levels in water fluctuate widely depending on a number of seasonal and climatic factors (20) as illustrated by the variation in Al^{+3} content of Tennessee's surface waters (21). As a first approximation to an experimental dose we determined the Al^{+3} content of some common beverages sold in bottles and cans. Considering the multiple sources of chronic Al^{+3} exposure, an arbitrary experimental dose of 100μM was decided upon.

After one year the control and Al^{+3} fed rats were tested in a T-maze for learning and recall. Although the Al^{+3} fed rats tended to require more time and made more errors to reach the food source, the results were only on the borderline of statistical significance (data not shown). This is not surprising in view of the well-known resistance of rats to Al^{+3}-induced behavioral changes (22). The average weight of the control rats was 620.6 ± 24.4 and that of the Al^{+3} fed group was 598.6 ± 25.5 g. The average weight of the control and Al fed livers was 21.4 ± 3.4 g and 21.6 ± 2.8 g respectively. The average weight of the brains of the control and Al fed rats was 1.91 ± 0.14 g and 1.99 ±

0.11 g respectively. There were no significant differences between the two groups for body, liver and brain weights.

Consistant with the earlier observations (23) the brains of the Al^{+3}-fed group contained more Al^{+3} (data not shown) but more importantly some of this was sequestered by ferritin (table 1.1). Brain ferritin from the control group contained 42.1 ± 14.3 g atoms of Al^{+3} per mole of ferritin, but that from the Al^{+3} fed group contained 2.7 times more ($p < 0.01$). The higher ferritin content of the brains from the Al^{+3} fed rats was statistically insignificant.

The liver ferritin from both the control and Al^{+3} fed rats contained far less Al^{+3} than the corresponding brain ferritin from each group. Additionally, 1.4 times more Fe was bound to the ferritin from the brains of the Al^{+3}-fed animals ($p < 0.05$). In contrast with brain ferritin, the total liver ferritin from the Al^{+3}-fed animals was 1.5 times more than that from the controls ($p< 0.02$). Therefore, in rats, increased Al^{+3} intake produced an increase in Al^{+3} and Fe bound to brain ferritin and more liver ferritin appears to have been synthesized.

In view of the suspected role of Al^{+3} in the aetiology of Alzhemier's disease and encouraged by the differences in Al^{+3} content of the ferritin in rat brain and liver, we isolated ferritin from the brains of two AD patients and

Table 1-1. Effect of Long-Term Al^{+3} Feeding on Rat Ferritins. For details see the Materials and Methods section.

GROUP	TISSUE	FERRITIN (μg/g wet wt.)		Fe (g atom/mole)		Al^{+3} (g atom/mole)	
		ave	SD	ave	SD	ave	SD
CONTROL	LIVER (n = 5)	152.6	±31.2	1257.7	±276.4	4.6	±1.8
	BRAIN (n = 5)	49.1	± 9.5	1036.8	±350.3	42.1	±14.3
Al^{+3} FED	LIVER (n = 5)	241.6	±54.3[a]	1262.5	±287.1	4.3	±2.3
	BRAIN (n = 6)	53.7	± 8.8	1505.0	±326.5[b]	115.4	±48.3[c]

[a] Significantly greater than the control, ($P < 0.02$).
[b] Significantly greater than the control, ($P < 0.05$).
[c] Significantly greater than the control, ($P < 0.01$).

two non- demented patients and quantified its Al^{+3} and Fe content (table 1.2). Human brain contained a substantial amount of ferritin, almost 30% of that found in human liver, and the ferritin from AD brains contained 5.6 times more Al^{+3} than the controls ($p < 0.01$). In addition, the ferritin from AD brains contained 33% more Fe than the ferritin from patients without AD ($p < 0.05$). Although the total amount of isolatable ferritin from both rat and human brain averaged approximately 50 μg/g brain tissue, the greater mass of human brain tissue yielded much more ferritin for further characterization. The molecular weight of human brain ferritin was determined to be about 500,000 by gel filtration (data not shown). Upon electrophoresis, under non-denaturing conditions, ferritin from human brain, liver and horse spleen showed different mobilities (fig 1.1). All three preparations also contained a slower moving iron-containing minor band representing a dimer of ferritin. In SDS, human brain ferritin dissociated into H and L subunits of Mr 22,130 and 19,400 respectively (fig 1.2).

Densitometric scanning of the gels showed that the relative proportion of the H and L chains was approximately 70% and 30% respectively for both normal and AD ferritin (data not shown).

Ferritin displays microheterogeneity by resolving into

Table 1-2. Al^{+3} and Iron in the Ferritin from Normal and AD Human Brain. For details see the Materials and Methods section.

Source	Brain Wt. (g)		Ferritin (μg/g brain)		Al (g atoms/mole)		Fe (g atoms/mole)	
	ave	SD	ave	SD	ave	SD	ave	SD
Normal (n = 2)	1154.5±47.0		38.1±13.6		3.4±0.6		435.6±38.3	
Alzheimer's (n = 2)	1082.0±21.5		60.4± 8.3		18.9±0.4[a]		579.5±3.7[b]	

[a] Significantly greater than normal ($P < 0.01$).
[b] Significantly greater than normal ($P < 0.05$).

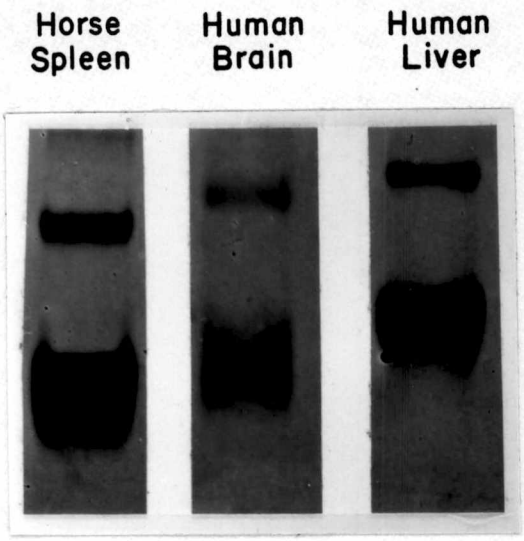

Figure 1.1. Native PAGE Electrophoresis of Human Brain, Human Liver and Horse Spleen Ferritins. For details see the Materials and Methods section.

Figure 1.2. Gradient SDS-PAGE Electrophoresis of Ferritin from Human Brain (Normal and AD). For details see the Materials and Methods section.

multiple bands upon isoelectric focusing. Two different interpretations of ferritin IEF bands have been offered. They have been explained as families of hybrid molecules consisting of differing proportions of the H and L chains (24) and methodological artifacts (25). While this controversy suggests that the IEF data should be treated cautiously, the differences between human liver and brain ferritin IEF patterns are striking. Five major and four minor acidic bands were visible for liver ferritin, all clustered in the pI range 4-5.5. In contrast, brain ferritin resolved into about twenty bands spread out over a much greater pI range, with only about five bands overlapping those of liver ferritin (fig 1.3). The major band was highly positively charged with an apparent pI of 8 and represented 30% of the total protein and iron of the applied sample. This IEF pattern was generally reproducible whether the sample was applied near the anode or cathode, though resolution of all the bands was better and the band corresponding to pI of 8 was more intense when applied near the cathode. A precise matching of the iron content with the individual isoferritin bands was not possible because the bands, though visually quite distinct, could not be adequately separated by the gel slicing procedure. An attempt to analyze the same gel slices for Al^{+3} was unsuccessful because of the minute levels of Al^{+3}

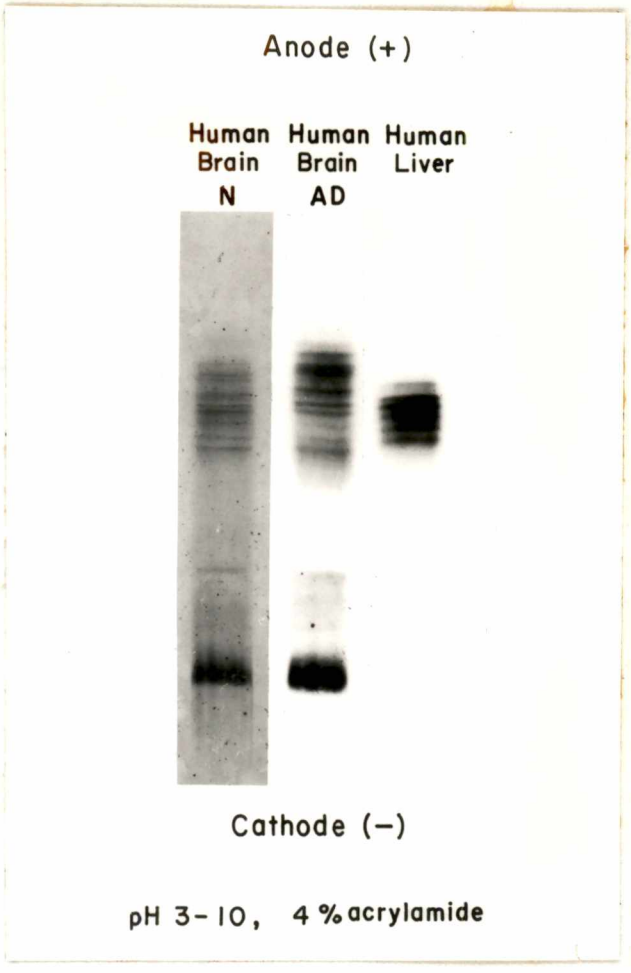

Figure 1.3. Isoelectric Focusing of Ferritin from Human Brain (Normal and AD) and Human Liver. For details see the Materials and Methods section.

associated with the isoferritins approached the limit of detection and the background contamination of the gel was too high.

The absence of an increased ferritin-Al complex in the livers of Al^{+3} fed rats and the presence of high levels of Al^{+3} bound to the brain ferritin from the control animals is significant. Although the IEF patterns of ferritin from human liver and brain were markedly different, inferences as to the physical significance of such a comparison must be made cautiously. Equilibrium dialysis of human brain, human liver and horse spleen ferritin against $AlCl_3$ with subsequent Fe, Al^{+3} and PO_4 determination showed that the increase in Al^{+3} bound to ferritin is correlated with an increase in PO_4 content. The PO_4/Al^{+3} ratio was similar for all three ferritins (table 1.3). Brain ferritin actually bound less Al^{+3} than did liver ferritin. This suggests that some explanation other than a structural difference must account for their <u>in vivo</u> Al^{+3} binding differences. The difference in the total Al^{+3} bound to the isolated liver and brain ferritins might be due to a difference in the availability of Al^{+3} to bind to ferritin. In addition to the species difference, the higher Al^{+3} content of rat brain ferritin compared to human brain ferritin is most probably due to the experimental Al^{+3} levels to which the rats were exposed. While the binding

Table 1.3. *In Vitro* Binding of Al^{+3} to Ferritin Isolated from Human Brain, Human Liver, and Horse Spleen. For details see the Materials and Methods section.

Source	Fe (g atom)	Al (g atom)	PO_4 (g atom)	PO_4/Fe	PO_4/Al
Human Brain	723	86	353	0.49	4.1
Human Liver	997	131	421	0.42	3.2
Horse Spleen	1625	173	833	0.51	4.8

experiment was performed with mixtures of isoferritins, the possibility of isoferritins differing in their Fe and PO_4 content and therefore having differential Al^{+3} binding properties cannot be excluded.

The increase in liver ferritin from the Al^{+3} fed rats is also a significant finding. Whether these elevated levels are due to a reduction in the turnover of the pre-existing ferritin or due to Al^{+3}-induced increase in the synthesis of new protein remains to be established. While it is well known that Fe induces ferritin synthesis (2), induction by other metal ions has not been demonstrated.

The increase in Al^{+3} bound to the ferritin from AD patients gives credence to the controversial reports of a correlation between increased Al^{+3} levels in certain parts of the brain and AD (26). It may be that the increase in Al^{+3} bound is a reflection of the general increase in the availability of Al^{+3} to the brains of AD patients.

The isolation of a ferritin-Al complex from brain strengthens the suggested role for ferritin in metal toxicity. Al^{+3} may be cleared from the liver as a normal process to protect liver enzymes from Al^{+3} toxicity (27). Therefore little Al^{+3} is available for binding to liver ferritin. The increase in Al^{+3} bound to brain ferritin may be a detoxification mechanism to protect the brain metabolism from Al^{+3} that cannot be cleared by another

mechanism. If, as we suggest, ferritin is a multi-functional molecule (7), the possible alterations in these functions may be influenced by the elevated levels of bound Al^{+3}.

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CHAPTER II

BRAIN FERRITIN - CHARACTERIZATION AND ROLE OF Al(III) IN FERRITIN FUNCTION

INTRODUCTION

The ubiquitous iron (Fe) storage protein ferritin (Mr ~480,000) is composed of 24 subunits of at least two classes, heavy (H) and light (L), with molecular weights of approximately 19,000 and 21,000 in mammals. The subunits form a protein shell which can store up to 4500 iron atoms per ferritin molecule as non-toxic ferric hydroxyphosphate (1).

In addition to storing iron, we have suggested that ferritin also participates in metal detoxification. Ferritin binds V^{+2} and Tb^{+3} <u>in vitro</u> and Cu^{+2}, Zn^{+2}, Pb^{+2}, Cd^{+2}, Be^{+2} and Al^{+3} <u>in vivo</u> (2-8). Rats, whose ferritin synthesis in liver was increased 5-fold by Fe^{+3} injection, survived otherwise toxic doses of Be^{+2} (3). Ferritin protected and reversed the <u>in vitro</u> inhibition of $Na^{+}K^{+}$ATPase, alkaline phosphatase, RNA polymerase and phosphoglucomutase by Be^{+2} (4,5). Ferritin isolated from the livers of rats that had been injected with Cu^{+2}, Zn^{+2}, Cd^{+2}, Pb^{+2}, and Be^{+2} had some of the injected metal bound to it (6).

There has been increasing concern over the rise in

Al^{+3} in water due to acid rain. Normally stored in the soil as insoluable aluminosilicates and aluminoxides, Al^{+3} leaching dramatically increases with a slight decline in water pH (9). Organisms that evolved in an environment with low Al^{+3} levels are now exposed to and must deal with this potential toxin.

Recent studies have implicated Al^{+3} in several central nervous systems disorders including amyotrophic lateral sclerosis (10), dialysis encephalopathy (11) and Alzheimer's disease (AD)(12). In brain tissue from AD patients, Al^{+3} has been found associated with the neurofibrillary tangles (13) and aluminosilicates have been found at the core of senile plaques (14). While it is not clear whether Al^{+3} is the primary cause of the neuropathology, a recent epidemiological/neurological study of 4,100 people in England found individuals who lived in areas with high Al^{+3} water levels had a 70% greater chance of developing the disease (15).

Previously we have shown that ferritin exists in significant concentration in brain tissue, that ferritin isolated from rats fed a low level of Al^{+3} in the diet have more Al^{+3} bound to ferritin compared to control animals, and the ferritin isolated from the brains of AD patients had more Al^{+3} bound compared to age-matched control patients (7). To further understand the role

ferritin may play in Al^{+3} toxicity, we have further characterized human and rat brain ferritin with respect to subunit composition, subcellular location, and the effect of Al^{+3} on the ability of ferritin to bind and release Iron.

MATERIALS AND METHODS

The water used was passed through two ion exchangers and had a resistance of greater than 1.5 M ohms. Ultra-pure HNO_3 was obtained from Baker. Metal standards used for atomic absorption spectroscopy were obtained from Sigma and were used within the period of the expiration date. Thioglycolic acid, Napthol Blue Black, HEPES and Tris were obtained from Sigma. Acrylamide and bis-acrylamide were obtained from BDH Chemicals (Poole, England). Chelex-100 was obtained from Biorad. Prestained molecular weight standards were obtained from Bethesda Research Laboratories. Nitrocellulose was obtained from Schleicher and Schuell. Peroxidase-labelled antibodies were obtained from Vector laboratories. NAD(P)H-FMN-oxidoreductase was obtained from Boehringer Mannheim (West Germany). The ferritin radioimmunoassay kit was obtained from Ramco laboratories. Nitrogen Zero grade was obtained from Volunteer Oxygen (Knoxville, TN) and passed over a heated copper coil to remove any residual oxygen. Freund's ajuvant was obtained from Gibco.

Unstripped rat brains were obtained from Pel-Freeze (Rogers, Ark) and kept frozen at -20 C. All other chemicals were of the highest purity obtainable. All glassware and plasticware was first rinsed with 2% ultra-pure HNO_3 followed by a rinse with double deionized water.

Isolation of Brain ferritin

Rat and human brain ferritin was prepared as previously described with the following changes (7). The 0-50% saturated $(NH_4)_2SO_4$ precipitate was dissolved in a minimal volume of 5 mM Na_2HPO_4 buffer (pH 7.0), applied to a 1.8 x 75 cm Sephacryl S-300 column and eluted with the same buffer. The fractions were assayed for protein and iron. The iron rich fractions were pooled, and concentrated with a 30,000 molecular weight cut-off filter (Amicon PM-30). One ml of the concentrated sample was layered on top of a step sucrose gradient composed of 2 ml of 80% sucrose followed by a sequential overlay of 4.5 ml each of 50, 35 and 25 % sucrose. The gradient was centrifuged at 100,000 x g for 2 hr in a Beckman 60 Ti rotor at 37,500 RPM at 5°C. The iron rich band at the 80-50% sucrose interface was collected, dialysed against 5 mM Na_2HPO_4 (pH 7.0) buffer and concentrated with a PM-30 filter. Ferritin concentrations were determined by multiplying the value obtained from the method of Lowry by

0.7, using bovine serum albumin as a standard (4).

Immunology

Polyclonal antisera was raised against purified human liver and brain ferritin in New Zealand White rabbits by subcutaneous injection (16). 2 mg of ferritin in 1 ml of 5 mM Na_2HPO_4(pH 7.0) buffer was thoroughly mixed with 1 ml Complete Freund's Adjuvant and injected at 6 separate sites on the rabbit's back. A booster injection of 2 mg was given six weeks after the initial ferritin injection. After one month blood was collected at two week intervals over a period of three months. The blood was allowed to clot for 30 min and the serum separated using Vacutainer tubes. The I_gG fraction was precipitated with 50% $(NH_4)SO_4$ and was resuspended in phosphate buffered saline (PBS) to the original serum volume.

Immunoprecipitation of concavalin A, human brain ferritin, and soybean ferritin with antisera made against human brain ferritin and soy ferritin was done on 1% agarose Ouchterlony plates in 0.1M Tris/citrate pH 8.3 buffer, using 8μL wells (16). After applying samples to the appropriate wells, the plates were left undisturbed at 4°C for 48 hr, washed with two changes of PBS, dried, stained with coomassie blue and destained.

Western blots

Human and rat brain ferritin were run in duplicates on 10-20% gradient SDS-PAGE gels (7), blotted onto nitrocellulose overnight at 150 mA using a Biorad electroblotter with a 0.2 M glycine, 0.025 M Tris, 5 M methanol transfer buffer. The nitrocellulose was removed from the apparatus, cut into two halves, and one half stained with 0.1% amido black in 45% methanol, 10% acetic acid and 45% water followed by destaining in the same solvent. The other half of the nitrocellulose was incubated for 2 hr at room temperature with 2% non-fat dry milk, 0.05% Tween-20, in Phosphate buffered saline (PBS) followed by rinsing 3 times at 10 min intervals with 200 ml 0.05% Tween-20 in PBS. The blot was then incubated with 10 ml primary antibodies diluted 1/200 in a heat sealable bag for 12 hr at room temperature. The blot was then rinsed 3 times with 200 ml of 0.05% Tween-20 in PBS at room temperature followed by an incubation for 1 hr at 37°C in a waterbath with secondary antibodies (peroxidase labelled) diluted fifty-fold in PBS. Again the blot was rinsed 3 times 200 ml in 0.05% Tween-20 in PBS at room temperature, and developed in 30 ml 3.5 mM 4-chloro-1-napthol, 0.015 M H_2O_2. After development the reaction was stopped by flushing with water and the nitrocellulose dried between two sheets of Whatman 3 MM

filter paper. The blot was stored wrapped in alumiunum foil to prevent bleaching.

Al^{+3} loading

Metal-free holo-human brain ferritin was prepared by dialysis against 2 g Chelex in 1 L of 20 mM HEPES (pH 5.0) buffer. Metal-free apo-human brain ferritin was prepared by reduction with 1% thioglycolic acid in $NaCH_3COOH$ (pH 4.8) buffer followed by dialysis in 20 mM HEPES (pH 5.0) buffer (17). The demetallo-ferritins were incubated with $AlCl_3.6H_2O$ in 20 mM HEPES (pH 5.0) to yield different Al^{+3}/ferritin subunit molar ratios. The samples were dialysed against the same buffer to remove unbound Al^{+3} and the actual Al^{+3} bound to ferritin was determined by atomic absorption (7).

Iron uptake

Several preparations of ferritin were made with varying ratios of Al^{+3}/ferritin subunit as described above. These ferritins were added to 0.1 M HEPES (pH 7.0) buffer and 0.05 M $Fe(NH_4)_2(SO_4)_2.6H_2O$ prepared in water degassed with N_2 was added in a total volume of 0.5 ml to give a 6 Fe^{+2}/ ferritin subunit molar ratio. The reaction was then allowed to proceed for 30 s (18). The rate of iron loading was then determined by measuring the change in absorbance at 310 nm over 30 s, and subtracting the rate obtained by the

autooxidation of Fe^{+2} alone.

Iron Release

The rate of iron release from several mammalian ferritins was determined using a modification of the method of Jones et al (19). A solution containing 2.5 mM FMN and 2.5 mM α,α-bipyridyl in 0.22 M HEPES (pH 7.4) buffer was pipetted into a cuvette and degassed for 30 s with N_2. NADH was added to a final concentration of 2.5mM along with 0.06 units of NAD(P)H-FMN oxidoreductase to give a final volume of 0.5ml and the cuvette was stoppered with parafilm and allowed to incubate for 1 hr at room temperature. The solution was then again flushed with N_2, 5-20 μg ferritin was added, and the increase in absorbance at 522 nm was monitored over a period of 10 min.

Iron loading

A solution of $Fe(NH_4)_2(SO_4)_2.6H_2O$ in water degassed with N_2 was added to 100 μg Ferritin in 0.05 M HEPES, 20 mM KIO_3, 80 mM $Na_2S_2O_3$ (pH 7.0) to give a 200 Fe/ferritin molar ratio (2). Several ferritin preparations with various amounts of iron loaded were made by sequentially adding 200 mole Fe/mole ferritin aliquots. 10 min after the addition of iron the protein was dialyzed against the same HEPES buffer and centrifuged for 2 min at 5000 x g at room temperature in a Brinkmann

centrifuge. The iron in the supernatant was quantified by atomic absorption and the protein in the supernatant measured by the method of Lowry.

Subunit analysis

Native PAGE and SDS-PAGE were performed as described previously (7). SDS gels were stained with coomassie and the resulting bands scanned with an LKB laser densitometer.

Subunit separation

A reverse phase HPLC procedure as described by Collawn et al. was used to separate human and rat brain ferritin subunits (20). One mg of Brain ferritin in 0.5 ml of water was dissociated by incubation with acetic acid (67%), thioglycolic acid (5%) in a final volume of 0.86 ml on ice for 16 hr followed by thorough dialysis against 0.1% trifluoroacetic acid (TFA) and lyophilization in a Savant Speed Vac. Apo-human ferritin prepared by reduction against 1% thioglycolic acid (pH 7.0) in the presence of 3 I.U. catalase and 3 I.U. bovine serum superoxide dismutase was also used as starting material for subunit isolation.

Dissociated ferritin subunits were resuspended in 0.1% TFA and injected onto a Vydac C_{18} reverse phase column connected to a Waters dual pump HPLC. Fractions were collected by monitoring at 220 nm. The solvent system employed was: solvent A - 0.1% TFA in water; solvent B -

0.1% in 80% acetonitrile; solvent flow rate 1 ml/min. Human brain subunits were eluted with a linear gradient of 45-55% B for 40 min followed by 55-90% B for 20 min. Rat brain subunits were eluted with a linear gradient of 35-50% B for 40 min followed by 50-75% B for 20 min. Fractions containing protein were lyophilized in a Speed Vac, re-chromatographed, and again lyophilized. Aliquots of concentrated samples were resuspended in 40 μl SDS sample buffer and electrophoresed alongside human and rat brain ferritin.

Electroelution of Rat Brain Ferritin Subunits

Rat brain ferritin subunits were separated on SDS-PAGE gels, stained with coomassie blue overnight, destained, and the bands corresponding to the H and M subunits were excised. The gel slices were washed for 2 hr with several changes of water after which the protein was electroeluted at room temperature over a 30 min period in 50 mM ammonium bicarbonate (pH 7.0) at 20 mA with a Isco electroeluter. The eluted samples in 200 μl of buffer were lyophilized in a Speed Vac, extracted and precipitated twice with ice-cold 5% acetic acid, 5% triethylamine in acetone, and centrifuged at 13,000 RPM on an American Scientific table top centrifuge at 5°C. The samples were then lyophilized on a Speed Vac.

Amino acid analysis

Ferritin subunits, isolated as described above, were hydrolyzed at 110°C for 21 hrs in evacuated sealed tubes containing 6M HCl, 0.01 M 2-mercaptoethanol. Hydrolysates were dried on a Speed Vac concentrator and subjected to chromatography on a Beckman 121-M amino acid analyzer using a Beckman 3-h single column system (21).

Al^{+3} precipitation

Varying amounts of a freshly prepared 10 mM $AlCl_3.6H_2O$ solution in water were added to 100 μg of apo and holo-ferritin in 20 mM HEPES (pH 6.0) in a final volume of 0.5 ml to give several Al^{+3}/ferritin subunit molar ratios. After 30 min at room temperature the samples were centrifuged in a Brinkmann centrifuge at 5000 x g for 2 min and the supernatant assayed for protein by the method of Lowry.

Sub-cellular distribution

Ten g of fresh human brain tissue was homogenized in 90 ml of ice cold 1mM MOPS(Tris), pH 6.6, 0.32 M Sucrose, 1mM $MgCl_2$ and fractionated by differential centrifugation using the method of Lai et al.(22). The fractions were assayed for ferritin by RIA, for iron by atomic absorption spectroscopy and for protein by the method of Lowry. Lactate dehydrogenase was used as an enzymatic marker for the cytosolic fractions (23, 24) and fumarase and pyruvate

dehydrogenase were used as markers for the mitochondrial fractions (25).

Carbohydrate Staining

100 μg of human brain ferritin and rat brain ferritin were run on SDS-PAGE gels and stained for carbohydrates using the method of Racusen (26). Horseradish peroxidase was used as a positive control.

RESULTS

SDS-PAGE shows that human brain ferritin resolved into H and L subunits in proportions of 70 and 30%, respectively. Rat brain ferritin dissociated into three subunits with molecular weights of 23,000 (H), 22,000 (M), and 20,000 (L) in proportions of 26, 42 and 30%, respectively (fig.2.1).

Western Blotting

To initially categorize human and rat brain ferritin subunits as to subunit class, we probed western blots from SDS gels with anti-human heart ferritin heavy chain and anti-human liver ferritin antibodies. While the putative human brain ferritin H chain reacted primarily with anti-human heavy chain antibodies and the putative L chain reacted primarily with anti-human liver antibodies, slight reactivity of the human H chain with anti-human

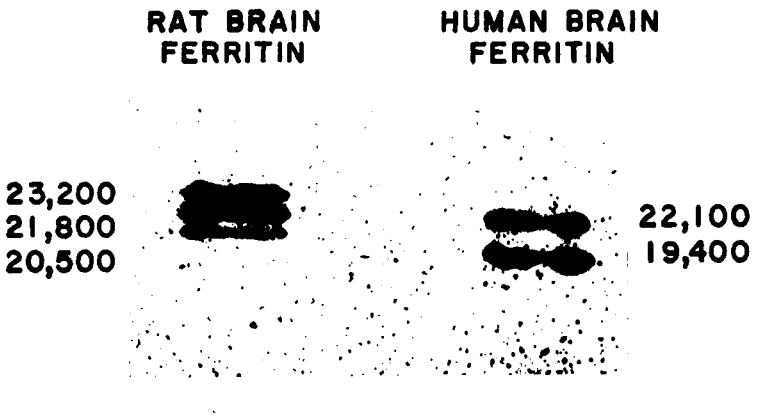

Figure 2.1. Gradient SDS-PAGE Electrophoresis of Ferritin From Rat Brain and Human Brain. For details see the Materials and Methods section.

liver antibodies was observed (fig. 2.2).

The rat brain subunits H and M (Mr 23,000, 22,000) showed no reactivity with either anti-human H chain or anti-human liver antibodies. The rat brain ferritin L subunit (Mr 20,000) showed reactivity with anti-human liver ferritin antibodies, but showed no reactivity with anti-human H chain antibodies (fig. 2.3).

Concavalin A, soybean ferritin and human brain ferritin were tested for immunoprecipitation against antisera raised against human brain and soybean ferritin. (fig 2.4.) Against anti-human brain antibodies there was no detectable precipitant band with soybean ferritin. Conversely there was no reaction with human brain ferritin against anti-soy ferritin antisera. Concavalin A showed no specific precipitant band with either plant or human antibodies though a diffuse non-specific precipitant band was observed at high concavalin A conentrations. A similar non-specific band was observed with concavalin A against antisera made to a yeast membrane protein.

Iron uptake

Several mammalian ferritins were compared for their iron uptake (table 2.1). The capacities of horse spleen and human liver ferritin were determined to be 3886 and 4147 mol Fe/mol ferritin, approaching the previously published value of 4500 mol Fe/mol ferritin (1). The

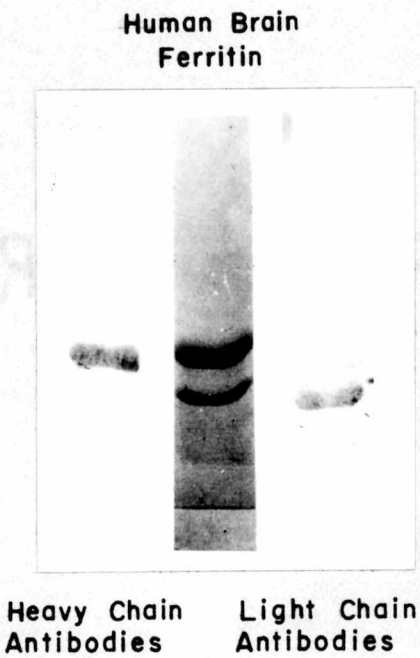

Figure 2.2. Western Blot of Human Brain Ferritin with Anti-Human Heavy Chain and Anti-Human Liver Ferritin Antibodies. For details see the Materials and Methods section.

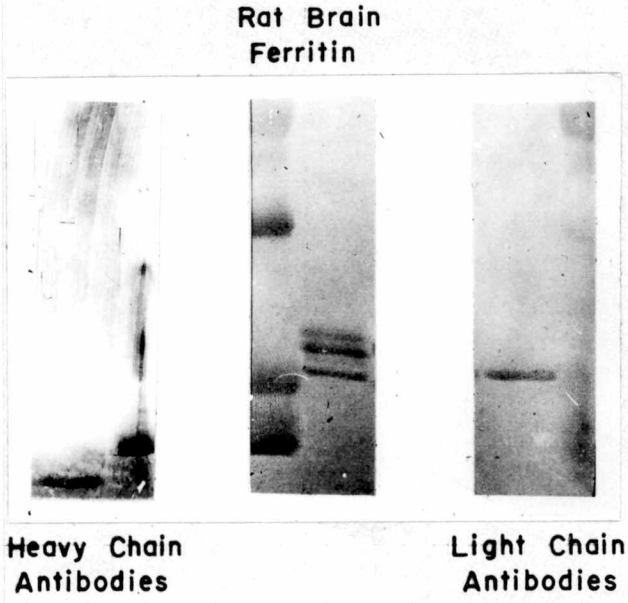

Figure 2.3. Western Blot of Rat Brain Ferritin with Anti-Human Heavy Chain and Anti-Human Liver Ferritin Antibodies. For details see the Materials and Methods section.

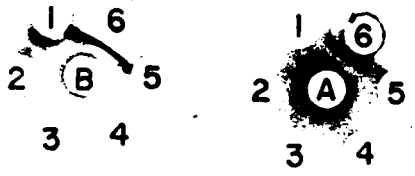

Figure 2.4. Immunoprecipitation of Concavalin A, Human Brain Ferritin and Soybean Ferritin with Anti-Human Brain and Anti-Soybean Ferritin Antibodies. A) rabbit anti -soybean ferritin antiserum, 8 μg; 1) Con A, 6.4 μg; 2) Con A, 3.2 μg; 3) Con A, 1.6 μg; 4) Con A, 0.8 μg; 5) human brain ferritin 0.8 μg; 6) soybean ferritin, 0.8 μg. B) rabbit anti-human brain ferritin, 2 μg; 1) Con A, 10 μg; 2) Con A, 5μg, 3) Con A, 2.5 μg; 4) Con A, 1.7 μg; 5) soybean ferritin 1.5μg; 6) human brain ferritin 1.5 μg.

Table 2.1. *In Vitro* Iron Uptake of Human Brain (AD), Human Brain (Normal), Human Liver and Horse Spleen Ferritins.

Ferritin	Initial Fe (mol Fe/mol ferritin)	Final Fe (mol Fe/mol ferritin)	Protein loss %
Horse Liver	2366	3886	48
Human Liver	2700	4147 ± 117	25
Human Brain (AD)	1661	2868	23
Human Brain (N)	1489	2616	24

[a]Each value represents the average of three individual determinations expressed as means ± Std. Dev. For details see the Materials and Methods section.

capacity of human brain ferritins obtained from normal or AD brain was less, averaging about 2700 mol Fe/mol ferritin. AD and normal human brain ferritin showed no significant difference in their iron loading capacity.

Iron Release

The rate of release of iron from several mammalian ferritins was compared to human brain ferritin (AD and normal)(table 2.2). In addition, several human brain ferritin preparations with varying Al^{+3}/subunits ratios were used to determine if Al^{+3} would affect the in vitro rate of iron release. As seen, Al^{+3} did not significantly affect the rate of iron release for either AD or normal human brain ferritin (fig.2.5).

Iron Loading

To determine if Al^{+3} would affect iron loading into human brain ferritin, several preparations of apo-human brain ferritin were preloaded with various Al^{+3}/subunit ratios and the in vitro rate of iron loading was determined (fig 2.6). Al^{+3} caused a concentration dependent decrease in the initial rate of iron loading into demetallo-apo-human brain ferritin. There was no difference between AD and normal human brain ferritin in the Al^{+3} affected decrease in iron loading.

Table 2.2. In Vitro Rate of Iron Release from Human Brain (Normal and AD), Rat Brain, Rat Liver, Human Liver and Horse Spleen Ferritins.

Ferritin	Fe initial (mol Fe/mol ferritin)	Vmax
Horse Spleen	1328	101.4 ± 15.9 (n = 4)
Rat Brain	1391	21.1 ± 4.0 (n = 4)
Rat Liver	1065	13.0 ± 7.2 (n = 3)
Human Liver	1750	33.4 ± 5.3 (n = 5)
Human Brain (AD)	1661	12.1 ± 2.6 (n = 10)
Human Brain (N)	1489	14.0 ± 2.7 (n = 10)

Vmax is expressed as nmol of Fe(II) released per min per nmol of initial Fe(III). Each value represents the average of five individual determinations expressed as means ± Std. Dev. For details see the Materials and Methods section.

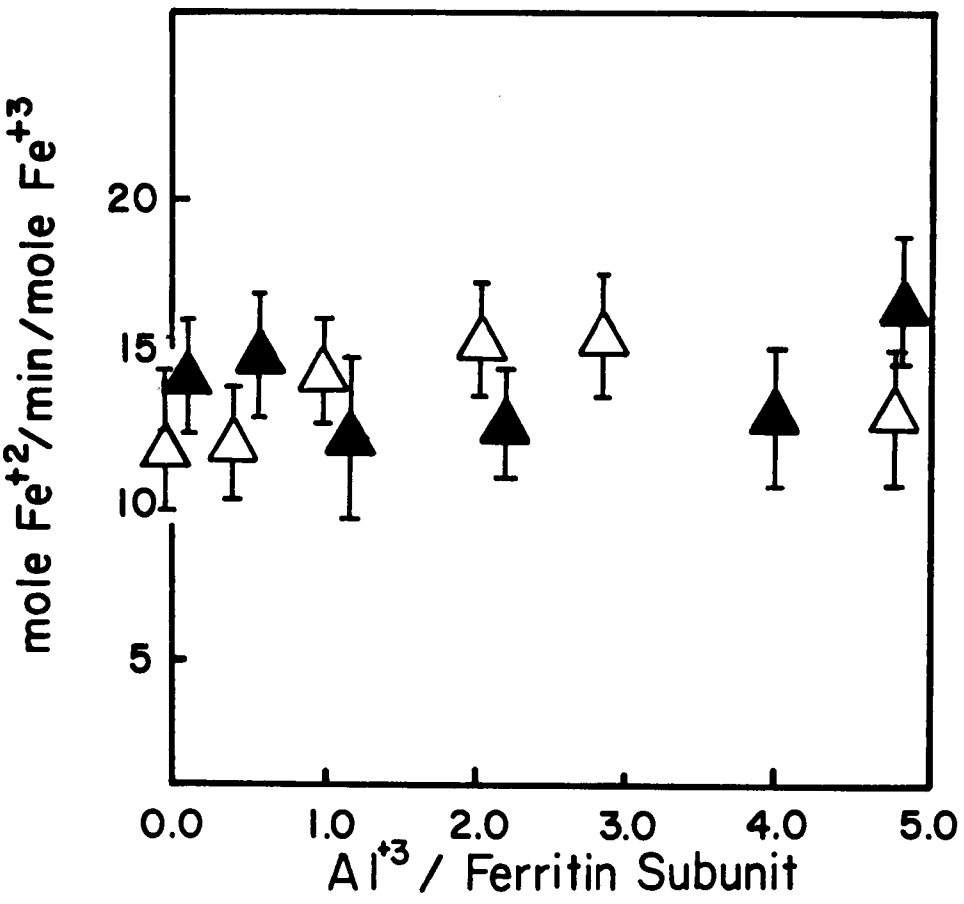

Figure 2.5. Effect of Al^{+3} on the Rate of <u>In Vitro</u> Iron Release from Human Brain Ferritin (Normal and AD). V_{max} is expressed as nmol of Fe(II) released per min per nmol of initial Fe(III). Each value represents the average of five individual determinations expressed as means ± Std. Dev. (△) human brain ferritin (AD); (▲) human brain ferritin (Normal). For details see the Materials and Methods section.

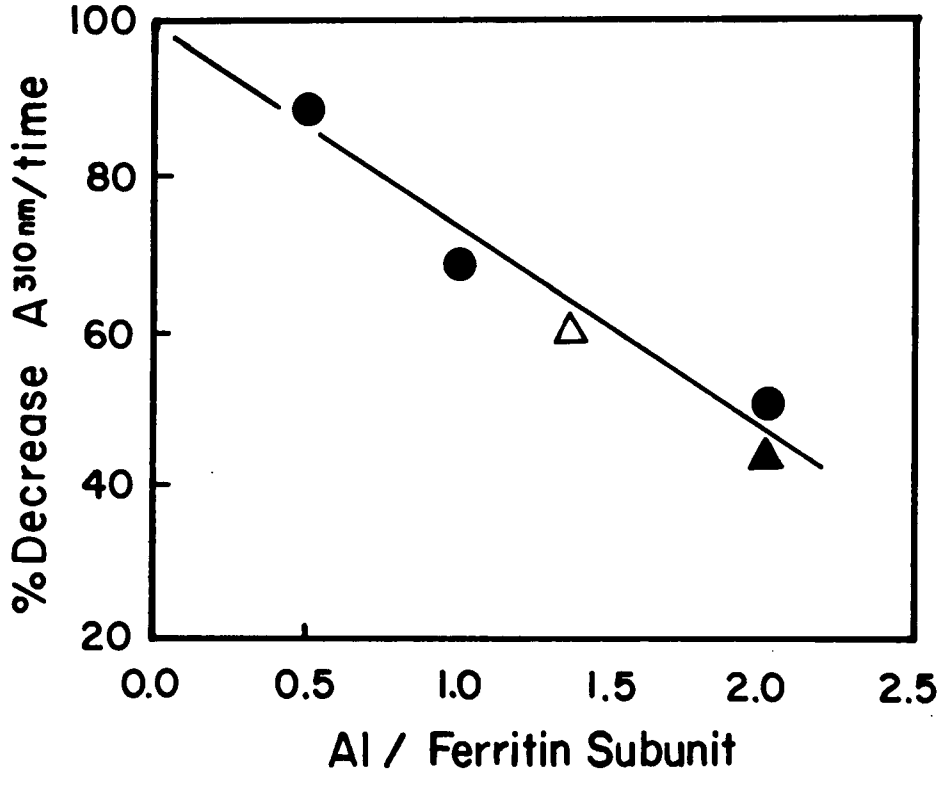

Figure 2.6. Effect of Al^{+3} on the In Vitro Rate of Iron Loading into Human Brain (AD), Human Brain (Normal) and Horse Spleen Ferritins. Values are plotted as a percentage of the rate of iron loading to ferritin without added Al(III). (▲) human brain ferritin (normal); (△) human brain ferritin (AD); (●) horse spleen ferritin. For details see the Materials and Methods section.

Ferritin precipitation

Aliquots of apo- and holo-human brain ferritin were challenged with increasing concentrations of $AlCl_3.6H_2O$ and the percent precipitated protein was determined at each Al^{+3} concentration (fig. 2.7). Holo-ferritin was more resistant to precipitation by Al^{+3} compared to apo-ferritin.

Isolation of Subunits

HPLC chromatography of human brain ferritin subunits (fig. 2.8) showed a number of major and minor peaks which upon subsequent SDS-PAGE were categorized as to L or H chain class. Seven H chain peaks were observed compared to five L chain peaks. While one major L chain peak representing 74 % of the total L chain protein was observed, the two major H chain peaks represented approximately 24 and 41 % of the total H chain protein. The cluster of H chain peaks migrated with an SDS R_f value identical to that of the human brain ferritin H chain (fig. 2.9). Four H chain class peaks were isolated and rerun on SDS-PAGE giving slightly different R_f values.

The HPLC elution profile of the H chain subfractions was generally reproducible for different ferritin preparations with similar elution times for the major peaks upon re-chromatography. Some variation in peak area was observed from preparation to preparation and there was no correlation of these differences in peak area between

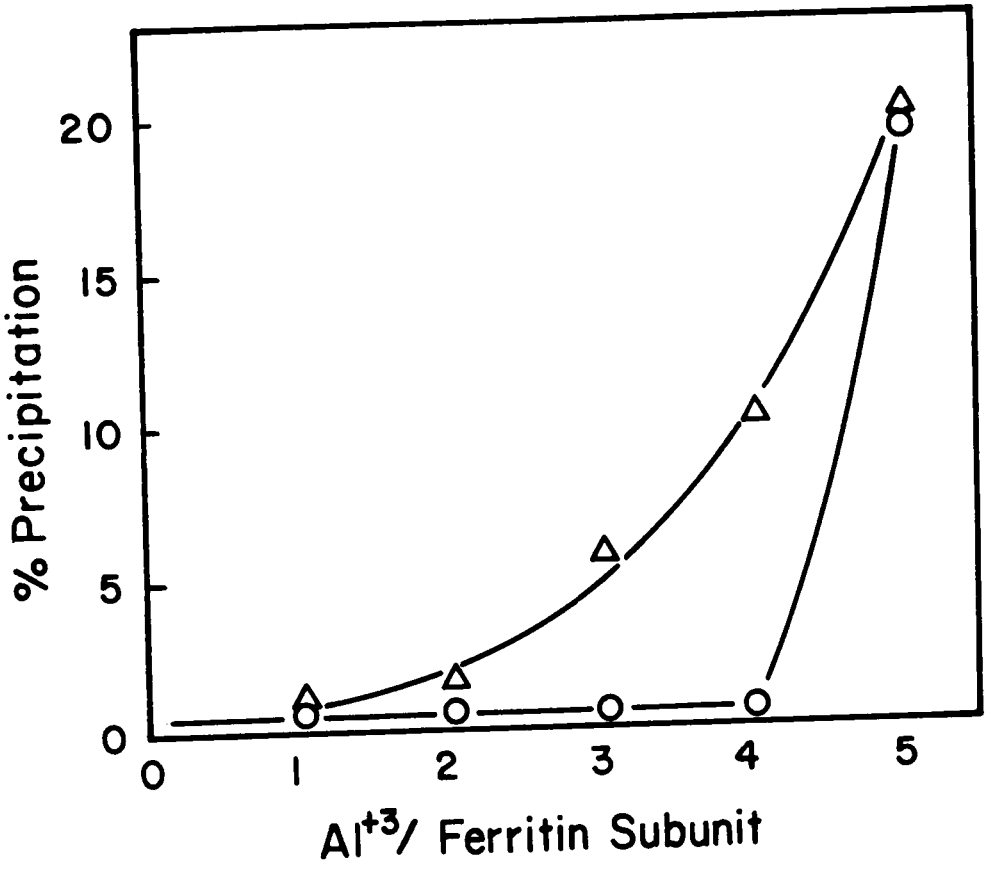

Figure 2.7. Effect of Al^{+3} on the In Vitro Precipitation of Horse Spleen Ferritin. Values are expressed as the percent of the original protein precipitated. (○) holo-horse spleen ferritin; (△) apo-horse spleen ferritin. For details see the Materials and Methods section.

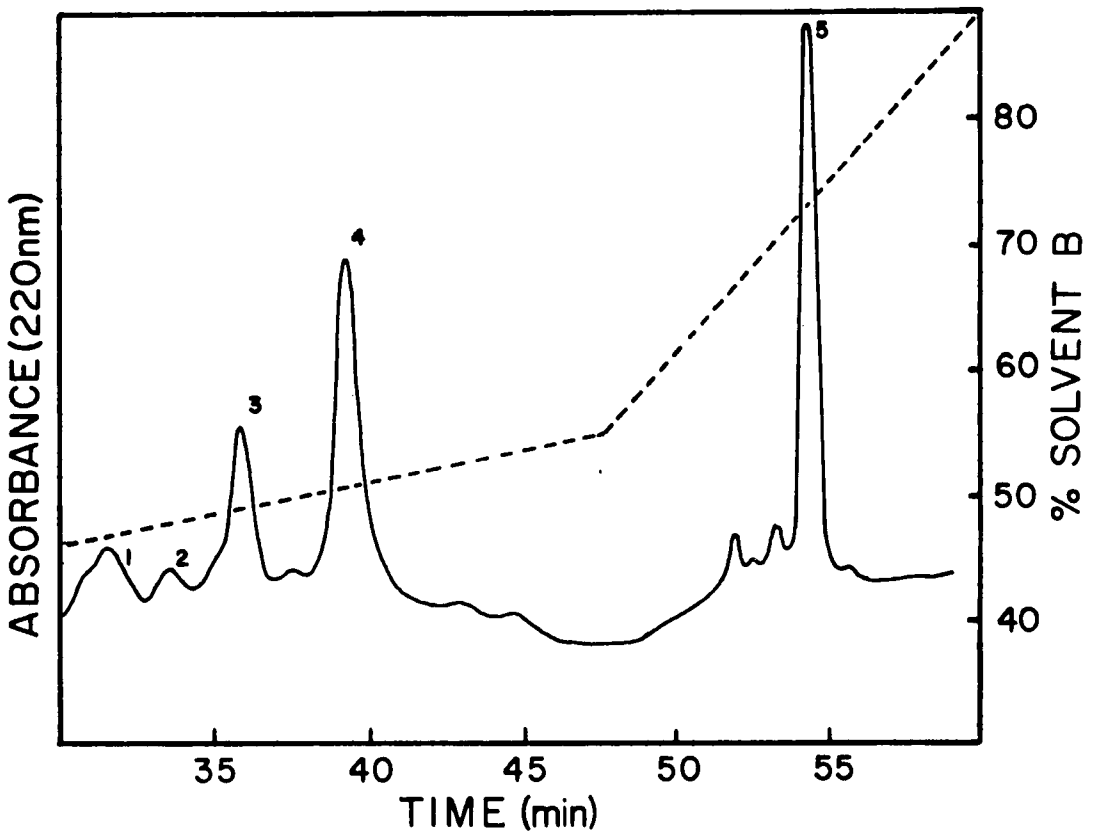

Figure 2.8. Reverse Phase HPLC of Human Brain Ferritin Subunits. Details are described in the Materials and Methods section. Eluted fractions were monitored at 220 nm. Solvent A: 0.1% TFA, Solvent B: 0.1% TFA, 80% Acetonitrile, Gradient: 45-55% B 45min, 55-90% B 20 min.

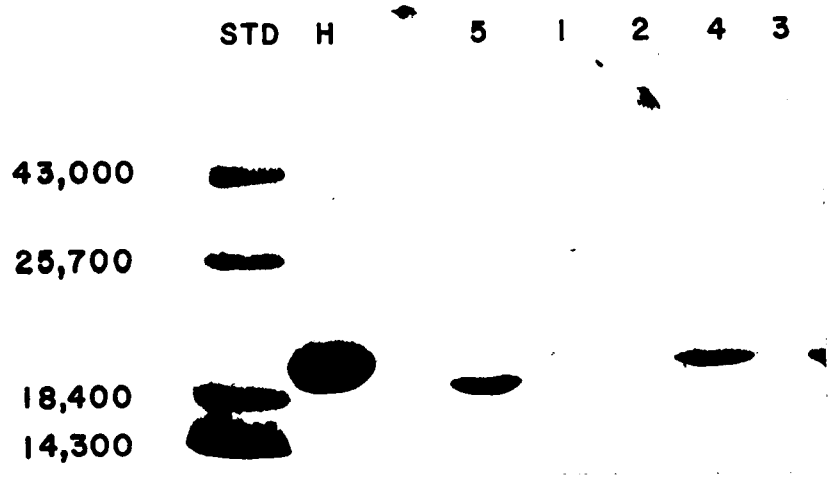

Figure 2.9. Gradient SDS-Page of Human Brain Ferritin Reverse Phase HPLC Subunit fractions. Details are given in the Materials and Methods section. Molecular weight standards (STD), combined human brain ferritin HPLC peaks 1-4 (H). Numbers refer to human brain ferritin HPLC fractions in fig. 2.8.

ferritins prepared from several normal or AD brains (data not shown).

HPLC chromatography of rat brain subunits was qualitatively similar to that of human brain, though less complex (fig. 2.10). Six H chain peaks and two L chain peaks were observed with the major H chain peak representing 67% of the H chain protein and the major L chain peak representing 97% of the L chain protein. When several isolated H chain peaks were rerun on SDS-PAGE, both resolved into H and M subunits (fig. 2.11). Subsequent attempts to separate the rat brain H and M subunits with HPLC were unsuccessful.

Electroelution

The rat brain ferritin H and M subunits were eluted from stained SDS-PAGE gels and were extracted with 5% acetic acid and 5% triethylamine to remove SDS and coomassie blue. The extracted pellet, still retaining some blue color would not dissolve in 0.1% TFA precluding further purification on HPLC.

Amino Acid Analysis

Two human brain ferritin HPLC H chain peaks and and one L chain peak were purified to homogeneity and their amino acid composition was determined. The values so obtained were compared with the predicted amino acid composition

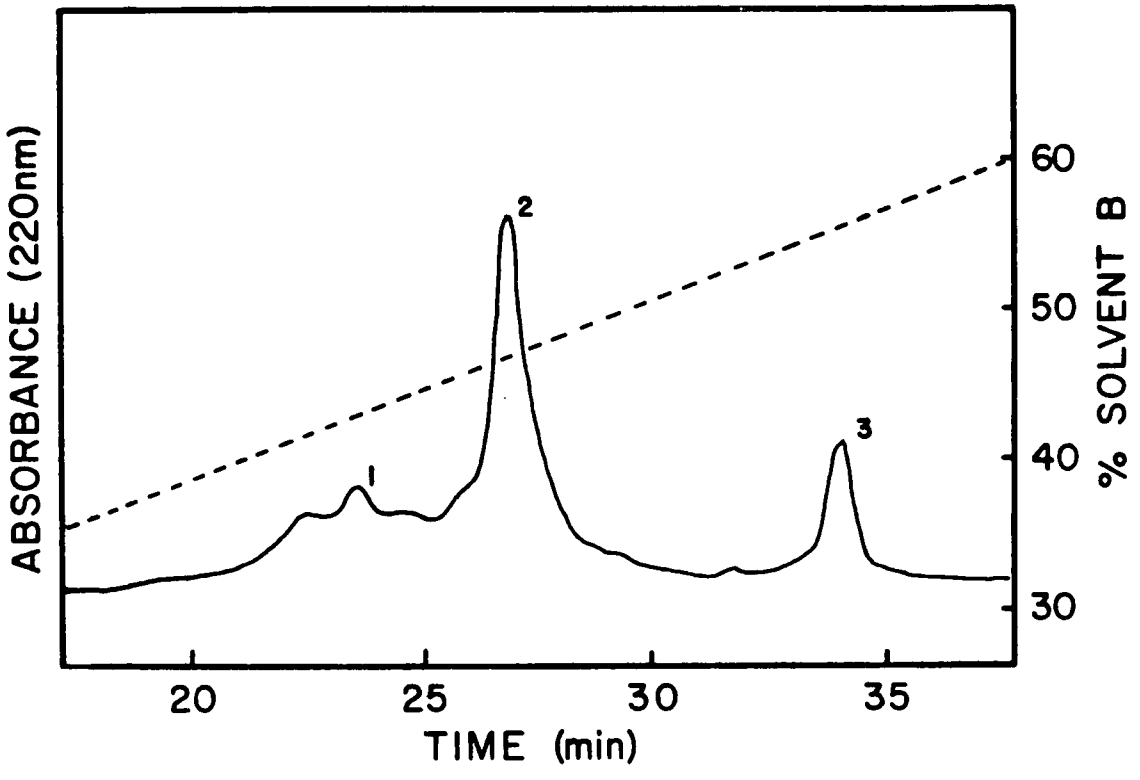

Figure 2.10. Reverse Phase HPLC of Rat Brain Ferritin Subunits. Details are described in the Materials and Methods section. The solvent system employed was: Solvent A: 0.1% TFA, Solvent B: 0.1% TFA, 80% Acetonitrile. Samples were eluted with a linear Gradient of 40-90% B for 75 min and fractions were collected and monitored at 220 nm.

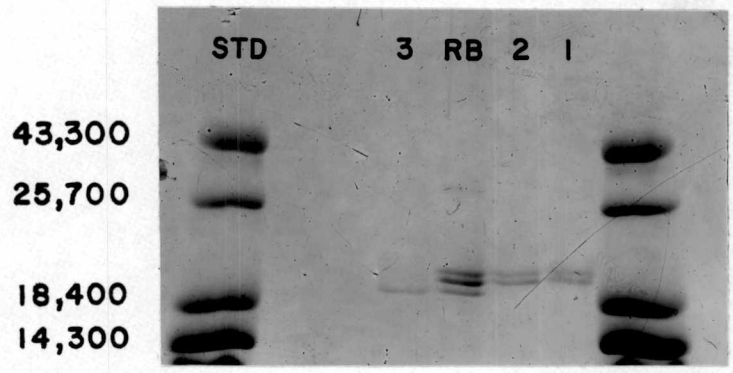

Figure 2.11. Gradient SDS-PAGE of Rat Brain Ferritin HPLC Subunit Fractions. Details are given in the Materials and Methods section. Molecular weight standards (STD), Rat Brain Ferritin (RB). Numbers refer to rat brain ferritin HPLC fractions as shown in Fig. 2.10.

deduced from the published cDNA sequences for the human liver H and L chains (44)(table 2.3). A comparison of the two human brain ferritin H chain HPLC peaks shows that their amino acid compositions are essentially identical. While the comparison of the human brain and liver ferritin subunits suggests that their amino acid compositions are very similar, some differences are significant. The human liver L chain has 1 valine residue in comparison to the human brain L chain which has 6.5 ± 0.4 valines. In addition, the human liver H chain has 15 lysine residues while the human brain H chain peaks have 11.4 ± 0.2[a] and 11.3 ± 0.6[b] lysines, respectively.

Similarly, amino acid composition data obtained from the hydrolysis of the rat brain ferritin HPLC L chain and the electroeluted rat brain H and M chains were compared with the amino acid composition deduced from cDNA clones for the rat liver H (45) and L (46) subunits, respectively. As illustrated in table 2.4., the rat brain L chain and the rat liver L chain are very similar with several notable exceptions. The rat liver L chain has 17 predicted glx and 21 arg residues compared with 28.7 ± 2.1 glx and 15.1 ± 0.3 arg residues observed in the rat brain L chain. While a comparison of the rat brain ferritin H and M chains suggests that they are nearly identical in amino acid composition, both differ siginificantly from the deduced H

Table 2.3. Amino Acid Composition of Human Brain Ferritin H and L Subunits Compared with Human Liver Subunits. For details see the Materials and Methods section.

Amino Acid	Number of Residues per Subunit				
	Human liver	Human brain	Human liver	Human brain	Human brain
	L chain	L chain	H chain	H chain[a]	H chain[b]
Lys	12	11.4 ± 0.6	15	11.4 ± 0.2	11.3 ± 0.6
His	7	6.5 ± 0.2	10	8.7 ± 0.7	8.9 ± 0.2
Arg	10	9.6 ± 0.1	7	6.4 ± 0.2	6.5 ± 0.2
Asx	19	19.1 ± 0.2	27	27.3 ± 0.3	27.4 ± 1.2
Thr	7	6.5 ± 0.1	7	6.6 ± 0.4	6.9 ± 0.1
Ser	8	7.6 ± 0.1	12	13.5 ± 2.1	10.9 ± 0.3
Glu	23	21.5 ± 1.8	26	27.6 ± 0.4	26.9 ± 0.7
Pro	4	4.1 ± 0.3	3	3.3 ± 0.5	3.1 ± 0.4
Glx	12	11.6 ± 0.4	7	8.1 ± 0.2	7.8 ± 0.1
Ala	15	15.0 ± 0.4	13	13.4 ± 0.2	13.8 ± 0.2
Val	1	6.5 ± 0.4	7	5.6 ± 0.1	5.6 ± 0.2
Met	5	3.8 ± 0.1	5	3.7 ± 0.1	4.3 ± 0.3
Ile	3	3.2 ± 0.2	6	5.5 ± 0.7	6.6 ± 0.1
Ieu	22	26.8 ± 1.3	22	21.9 ± 0.5	22.6 ± 0.1
Tyr	7	7.1 ± 0.1	8	9.1 ± 0.5	9.3 ± 0.3
Phe	8	7.7 ± 0.2	5	6.5 ± 0.1	6.0 ± 0.3

[a] Human brain HPLC subunit fraction 3 in fig. 2.10.
[b] Human brain HPLC subunit fraction 4 in fig. 2.10
Values are expressed as means ± Std. Dev. from two separate determinations.

Table 2.4. Amino Acid Composition of Rat Brain Ferritin H, M and L Subunits Compared with Rat Liver Subunits. For details see the Materials and Methods section.

Amino Acid	Number of Residues per Subunit				
	Rat liver	Rat brain	Rat liver	Rat brain	Rat brain
	L chain	L chain[a]	H chain	H chain[b]	M chain[c]
Lys	10	11.2 ± 0.6	13	12.9	11.8
His	7	6.4 ± 0.5	11	5.4	4.6
Arg	21	15.1 ± 0.3	9	10.3	8.4
Asx	18	18.2 ± 1.2	24	21.4	19.6
Thr	10	8.0 ± 0.1	6	11.4	10.9
Ser	8	8.7 ± 0.1	13	14.8	14.0
Glx	17	28.7 ± 2.1	25	24.0	22.8
Pro	3	6.7 ± 1.5	4	9.6	9.5
Gly	12	15.0 ± 0.7	8	21.7	17.7
Ala	17	15.8 ± 1.3	12	21.6	20.4
Val	9	9.0 ± 0.5	6	10.5	10.6
Met	3	2.5 ± 0.7	5	2.8	2.9
Ile	2	3.2 ± 0.5	6	8.2	7.6
Ieu	29	29.8 ± 1.5	21	19.2	19.7
Tyr	4	2.8 ± 0.1	8	8.2	7.5
Phe	9	7.0 ± 2.3	6	10.9	10.6

[a] Rat brain HPLC subunit fraction 3 in fig. 2.11.
[b] and [c] were obtained by electroelution from SDS-PAGE gels as described in Materials and Methods. L chain values are expressed as means ± Std. Dev. from two separate determinations. H and M chain values were obtained from a single determination.

chain composition in his, thr, pro, gly, ala, and val residues.

Subcellular Location

We isolated five crude human brain subcellular extracts and assayed each fraction for iron and ferritin (table 2.5). Brain ferritin was found primarily in the cytosolic fraction (58%) although significant amounts were also found in the cellular debris (19.3%) and microsomal fractions (15.6%). Most of the iron was found in the cellular debris (60%) and microsomal (26%) fractions.

Carbohydrate Analysis

To determine the presence of any carbohydrate moieties, SDS-PAGE gels of human and rat brain ferritin were stained for carbohydrates. No carbohydrate staining bands were seen for either human brain or rat brain ferritin (data not shown).

DISCUSSION

SDS-PAGE indicates that human brain ferritin is composed of two subunits (7), in agreement with the postulate first proposed by Drysdale that tissue ferritins are composed of various proportions of the H and L subunit types (27). To initially verify the homology of the human brain ferritin H and L chains with the respective human liver chains, we probed the human brain ferritin subunits

Table 2.5. Distribution of Ferritin and Iron in Human Brain Homogenates. For details see the Materials and Methods section.

Fraction	Ferritin (%)	Iron (%)
Cell Debris	19.3	60.0
Nuclear	1.7	1.4
Mitochondrial	5.4	9.4
Microsomal	15.6	26.0
Cytosolic	58.0	3.6

with anti-human ferritin H chain and anti-human liver ferritin antibodies. The finding that the putative brain H chain reacted with H chain antiserum and the putative L chain reacted with anti-liver antibodies indicated that, indeed, the two human brain ferritin subunits are of the H and L chain class. While there was no cross reactivity of the L chain with H chain antibodies, some cross reactivity was observed between the H chain and anti-liver antibodies. Since liver ferritin is composed of both L and H chains in a ratio of 9:1 (48) some cross reactivity is to be expected.

Because SDS-PAGE of human brain ferritin gave two bands, we were surprised to find that rat brain ferritin resolved into three distinct bands on SDS. We probed SDS-PAGE gels of rat brain ferritin with the human anti-H chain and anti-liver ferritin antibodies presuming that they would react with subunit specificity on the basis of the reported homology between the human and rat ferritin subunits (28). The fact that the rat brain L subunit reacts with anti-human liver antibodies, but not human H chain antibodies suggests that it be classified in the L chain class. In addition, the fact that rat brain ferritin subunits H and M do not react with anti-human H chain antibodies, and that rat brain subunit L does react with anti-human liver ferritin antibodies suggests that

while the rat brain third subunit is homologous with the human L chain, the rat brain subunits H and M are not homologous with the human H chain.

The similarity between the amino acid composition of the rat brain and rat liver L chains supports the immunological data for the L subunit. While the rat brain H and M subunits have nearly identical amino acid compositions, they are quite different in composition compared with the rat liver H chain, which may explain the lack of cross reactivity between the rat brain H and M chains and anti-human H chain antibodies. These two lines of evidence suggest that the rat brain ferritin H and M chains may have a distinctly different primary sequence compared to the rat liver H chain.

Concavalin A (Con A) is reported to have an apo-ferritin like oligomeric form into which iron can be deposited (39). Therefore it was tested in immunodiffusion experiments with human brain ferritin and soybean ferritin against anti-human brain and anti-soybean ferritin antibodies. No shared antigenic determinants were seen between the human and plant ferritin. This was in agreement with similar experiments which used soybean ferritin against anti-rat liver ferritin antibodies (40). This indicates that there is little conserved surface sequence homology between the plant and human proteins. Similarly, the lack of a

specific immunological reaction between Con A and anti-soybean ferritin antisera suggests lack of conservation between Con A and soybean ferritin. The non-specific reaction of Con A with IgG due to saccharide binding sites on the surface of the molecule limits the application of immunological techniques to the study of Con A, but Yamauchi et al. have shown that it is possible to distinquish between non-specific and specific reactions on double immuno-diffusion plates (41). Our subsequent computer homology analysis of Con A with rat, human and horse ferritin sub-units showed no conservation of sequence.

The finding of a third ferritin subunit is not without precedent. Watanabe, et al. found evidence for two classes of H subunits in rat liver and HeLa cells using 2D gels (29). Dickey et al. reported the cloning of a third subunit from embryonic tadpole erythrocytes (30). Collawn et al. isolated and sequenced two separate porcine liver H subunits (20).

The third ferritin subunit suggests either the expression of a third ferritin gene or a post-translational modifcation of the expressed subunits. Two separate genes for the H and L chains in rats (31) and humans (32) have been reported and multiple gene copies have been found in both species. However, they are presumed to be psuedogenes because they lack the introns found in expressed genes

(28). Perhaps the most compelling evidence for the existence of additional ferritin genes comes from the comparison of the cDNA deduced sequence and that obtained by direct protein sequencing. Two H chains were isolated from porcine spleen with two amino acid differences in the C terminal region suggesting polymorphism within the H chain gene (20).

The differences in the amino acid composition found in comparing the human liver ferritin H and L chains with the respective human brain ferritin chains also suggest possible sequence polymorphisms. Subunits were also prepared from apo-ferritin as a control for possible free-radical damage during subunit isolation. The human brain holoferritin was slowly reduced in the presence of catalase and superoxide dismutase to inhibit the propagation of free-radicals during the reduction of Fe^{+3} to Fe^{+2}. Subsequent subunit isolation from these apoferritins gave an HPLC elution profile identical to that with subunits prepared directly from holoferritin.

In a comparison of the isoelectric focusing profiles of human brain and and human liver ferritin, we previously observed a much wider P_I range for human brain ferritin. In addition to possible amino acid sequence differences mentioned above, another explanation for this effect might be a covalently attached carbohydrate, as has been

reported for serum ferritins (33). However, the staining of SDS-PAGE gels of human brain and rat brain for carbohydrates gave negative results. Multiple isoelectric bands might be due to protein deamination during isolation or an artifact of the isoelectric focusing procedure.

Studies on the kinetics of iron uptake by apoferritin have shown that hyperbolic curves were obtained when low Fe/ferritin ratios were used. The hyperbolic curve represents the initial phase of iron binding at specific sites within the apoferritin shell, after which the bound Fe^{+2} is oxidized to Fe^{+3}. Several investigators have reported that the cations Zn^{+2}, Ni^{+2}, Hg^{+2}, Cd^{+2}, Co^{+2} and Mg^{+2} caused inhibition of iron uptake by competing for the active site (2,8). Our results show that Al^{+3} binds to apo-human brain ferritin and inhibits iron loading in proportion to the amount of Al^{+3} bound. While the formation of an Al(α,α-dipyridyl)$_3$ complex as a possible artifact in the assay system has been suggested, such a complex decomposes immediately in aqueous solution (42).

Jones et al. found a 5 fold increase in the rate of dihydro-FMN mediated iron release from horse spleen ferritin compared to horse heart ferritin. They concluded that the rate determining step in the release of iron was most probably the diffusion of the dihydroflavin through the protein channels. They suggested that the decreased

iron release rate of horse heart ferritin may be due to a greater restriction of dihydroflavin diffusion to the iron core caused by the presence of larger H subunits compared with the smaller L subunits of horse spleen ferritin. Our iron release data for human brain ferritin and human liver ferritin is consistent with this hypothesis; human brain ferritin (70% H-chain) has a V_{max} less than one-half that of human liver ferritin. However, a comparison of the iron release rates for rat brain and rat liver ferritin reveals that rat brain, with 26% H, 42% M and 30% L has a 65% greater V^{max} compared to rat liver ferritin which is 62% L chain (47). While this result contradicts Jones' model, rat brain ferritin with its three subunits may present an unusual case in which the three dissimilar subunits form larger channels than those formed in rat liver ferritin. The differences in the observed rates of iron release among the mammalian ferritins studied emphasizes the importance of research in ferritin tissue heterogeneity and in particular, the accumulation of data on tissue specific ferritins.

The fact that apoferritin is more susceptible to Al^{+3} precipitation compared to holoferritin suggests that while there are Al^{+3} binding sites on the exterior of the protein that permit aggregation, Al^{+3} preferentially binds to the ferritin iron core. This finding prompted us to determine if bound Al^{+3} would interfere with the rate of

iron release from ferritin, and if the effect would be different for ferritin isolated from AD or normal brains. Pre-loading human brain ferritin with Al^{+3} did not significantly affect the <u>in vitro</u> rate of iron release from ferritin obtained from either AD or normal brain tissue. Apparently Al^{+3} binds to the ferric hydroxyphosphate core in such a way as not to interfere with the Fe^{+3} reduction reaction.

The finding that ferritin is found predominantly in the cytosol is in general agreement with previous reports and isolation schemes. That significant concentrations of ferritin are also found in the cellular debris, nuclear and microsomal fractions is perhaps more controversial. Several early ultrastructural studies have reported nuclear and mirochondrial ferritin in iron loaded liver cells (34,35). The occurance of 20% of the total ferritin in the cellular debris along with 60% of the iron may perhaps be due to the presence of insoluble hemosiderin. Confirmation of this hypothesis will require ultra-structural and histological studies analogous to those reported on human liver cells (36).

The finding of significant levels of iron in these different brain tissue subfractions emphasizes the role iron mediated free radical generated lipid peroxidation may play in neuropathology. Our finding of a significant

decrease in iron release with as little as 0.5 Al/ferritin subunit, suggests that even the levels of Al^{+3} found associated with brain ferritin in vivo may adversely affect ferritin function. The alteration of ferritin iron storage, transport, and function by trace metals such as Al^{+3} may contribute to disorders in brain regions with high iron content such as the basal ganglia (37).

We reported an elevated Al content associated with ferritin from AD brains but we found that the isoelectric focusing pattern of normal or AD ferritin was very similar (7). In contrast, Broomhead et al. observed significant differences in the isoelectric properties of ferritins isolated from the two types of brain tissue with no Al^{+3} associated with either ferritin (38). This discrepancy may result from differences in the patients' stage or severity of the disease. Assuming comparable methods of Al^{+3} analysis, Broomhead's data suggests that the amount of Al^{+3} bound to ferritin may depend on the environmental Al^{+3} exposure of the individual.

The greater number of human brain isoferritins compared to other human tissue ferritins may be highly significant. The reported increase in acidic isoferritins in Chang cells after iron loading (37) and the implication of acidic isoferritins in the control of stem cell hemopoisis (38) suggests that isoferritins have different

functions in the cell. Changes in isoferritin profiles during development, such as an increase in basic isoferritins after gestation, suggest that isoferritins have an important cellular regulatory role in addition to their function in iron storage. If specific isoferritins are selectively localized in different brain regions, this would emphasize the critical iron regulatory reguirements of neural tissue. The effect of Al^{+3} in decreasing the rate of iron loading into brain ferritin may be more pronounced in some iosferritins than others. Non-ferrous metals including Al^{+3} may also affect the regulatory functions of brain isoferritins. Alterations in isoferritin function may therefore have adverse developmental consequences that would be disatrous in post-mitotic neurons, particularly in the aging brain. A greater understanding of brain isoferritins and their functions is therefore essential to understanding neural metabolic and developmental processes.

Slow deposition of Al^{+3} in the brain and its subsequent binding to ferritin would generate ferritin molecules less efficient in regulating iron. Preliminary experiments show that unlike iron, Al^{+3} does not induce <u>de novo</u> synthesis of ferritin (unpublished observations). Therefore, elevated levels of ferritin observed in the brains of AD patients or Al^{+3}-fed rats may be an indirect consequence of Al^{+3}

toxicity where more functional ferritin molecules are required to sequester iron.

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CHAPTER III

IMPLICATIONS FOR FURTHER WORK

This dissertation has dealt with the isolation and characterization of human and rat brain ferritin. We chose to study the *in vivo* and *in vitro* effects of Al^{+3} on brain ferritin because this metal is a known neurotoxin and it is believed by many researchers to be involved in the etiology of Alzheimer's disease.

The ferritin isolated from rats fed 100 μM Al^{+3} had three times the Al^{+3} bound compared to control animals. In addition, while the liver ferritin from the Al^{+3} fed group did not have a significantly greater amount of Al^{+3} bound, there was 60% more liver ferritin in the Al^{+3} fed animals compared with the controls. While it is well known that iron induces ferritin synthesis, the question of whether non-ferrous metals are also inducers has only been superficially studied. Dr. R.E. Thatch at Washington University in St. Louis has found that 3 μM Al^{+3} citrate did not induce ferritin synthesis in cultured mouse cells (personal communication). Our data suggest that rigourous *in vitro* transcriptional and translational experiments should be undertaken to answer this question.

Ferritin isolated from the brains of patients who died of Alzheimer's disease had five times the Al^{+3} bound to

ferritin compared to aged matched control patients. The isoelectric focusing pattern of human brain ferritin was considerably different from that of human liver, with only 5 of the 20 brain ferritin bands migrating similarly to the liver bands. The greater number of isoferritins in the brain suggests that regulation of neural iron metabolism is much more complicated and perhaps more highly regulated than in other tissues. To further the understanding of brain isoferritins more information needs to be gathered about isoferritin profiles in specific brain regions by *in situ* mapping. In the interest of establishing more clearly the effect of Al^{+3} on brain ferritin function, *in vitro* iron loading and release experiments should be conducted with isolated isoferritins.

The finding of significant differences between the amino acid compositions of human brain and human liver subunits suggests that sequencing of the human brain ferritin gene is warranted. If upon sequencing, no differences between the brain and liver sequence are found, the mechanism of post-translational modification of brain ferritin should be investigated.

Besides the genetic interest in an expressed third rat brain gene, this finding also has implications for isoferritin structure and function. With three subunits the potential for 3251 isoferritins exists; why a particular

subset of these possible isoferritins exist should shed light on isoferritin function in general. Clearly, sequencing the rat brain genes should be a top priority.

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