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Factors Modulating Filament Formation by Bovine Glial Fibrillary Acidic Protein, the Intermediate Filament Component of Astroglial Cells†

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ABSTRACT: Glial fibrillary acidic protein (GFAP) is soluble in low ionic strength solutions but shows a strong tendency toward assembly with increasing ionic strength as revealed by electron microscopy and turbidity measurements. Increasing K^+, Na^+, and Li^+ concentrations cause an increase followed by a decrease in GFAP turbidity with a maximum at 200 mM, but their effects are much weaker than effects of divalent cations at the same ionic strength. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+} promote assembly at millimolar concentrations, and 10 μM Cu^{2+} causes rapid aggregation. The critical concentration for GFAP assembly was 0.08 ± 0.04 mg/mL in 2 mM Tris-HCl, 60 mM KCl, and 1 mM $CaCl_2$, pH 6.8. The M_r 38 000 rod domain of GFAP obtained by limited chymotryptic digestion is more soluble in 100 mM imidazole hydrochloride buffer, pH 6.8, than the intact molecule, and removal of the end pieces greatly reduces the ability of GFAP to form filaments. BNPS-skatole (2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine) treatment releases a M_r 30 000 N-terminus and a M_r 20 000 C-terminus. The M_r 30 000 polypeptide shows a higher affinity than the M_r 20 000 fragment for intact GFAP. Arginine and lysine at low concentrations slightly accelerate GFAP assembly, but above 100 mM both amino acids inhibit assembly. ATP, GTP, CTP, and UTP do not show significant effects on GFAP assembly. Dephosphorylation by alkaline phosphatase slightly reduces the assembly ability of GFAP, but phosphatase-treated GFAP still is assembly competent.

Intermediate filaments, distinguished by their 7–11-nm diameter and low solubility, are a major component of the cytoskeleton. On the basis of their immunological and biochemical properties, intermediate filaments are grouped into five subclasses: keratin, desmin, vimentin, neurofilaments, and glial filaments (Lazarides, 1980). These subclasses are distinct in their composition and cellular origins. Their polypeptide subunits, however, share a common structure: a conserved rod domain covering about 310 amino acid residues and flanking N-terminal and C-terminal domains (Geisler & Weber, 1982; Geisler et al., 1982). The rod domain contains extended α-helical regions which are able to form coiled coils and probably are responsible for the formation of the filament backbone (Steinert, 1978; McLachlan, 1978; Steinert et al., 1980).

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Differences among subunits mainly are due to the two end domains which are variable both in amino acid sequence and in length. These end domains may play roles in lateral interactions during filament formation and may aid connection with other filaments or cellular organelles (Steinert et al., 1983; Geisler et al., 1983a, 1984; Hanukoglu & Fuchs, 1983). Under physiological conditions, intermediate filaments are insoluble and must be extracted from detergent-insoluble pellets with denaturing reagents such as 8 M urea (Cook, 1976; Fukuyama et al., 1978; Rueger et al., 1979). Their subunits show remarkable propensities for assembly. Upon removal of denaturing reagents by dialysis, the polypeptide subunits assemble into filaments which biochemically and morphologically are very similar to those formed in vivo (Steinert et al., 1976; Osborn et al., 1977; Sun & Green, 1978; Rueger et al., 1979). High ionic strength and low pH favor filament assembly except for keratin filaments which are particularly prone to aggregate even in urea solution (Baden et al., 1973; Steinert, 1975; Fukuyama et al., 1978; Rueger et al., 1979; Moon et al., 1981; Jones et al., 1982).

Intermediate filament subunits are phosphorylated in vivo (Cabral & Gottesman, 1979; Lazarides, 1980; Browning & Ruina, 1984; McCarthy et al., 1985). The function of this phosphorylation still is unclear. However, data suggest that phosphorylation appears to modify the structure of the polypeptide subunits leading to changes in their immunogenicity (Julien & Mushynski, 1983; Garden et al., 1985; Georges et al., 1986), and recent work has shown a relationship between phosphorylation and subunit polymerization (Inagaki et al., 1987; Geisler & Weber, 1988).

Another common feature of intermediate filaments is their sensitivity toward Ca^{2+}-activated proteolysis which causes disintegration of intermediate filaments and collapse of the cytoskeletal framework (Zimmerman & Schlaepfer, 1982; Nelson & Traub, 1982). In certain epithelial and carcinoma cells transient formation of small, spheroidal aggregates of intermediate filaments can be observed without detectable increases in Ca^{2+}–protease-mediated degradation, suggesting the existence of additional cellular factors effective in disintegration and reorganization of intermediate filaments (Franke et al., 1982; Geisler & Weber, 1988).

Glial fibrillary acidic protein is the least studied component of intermediate filaments. It is specific to astroglia (Schachner et al., 1977; Eng & Rubenstein, 1978). In response to injury GFAP[1] accumulates in astrocytes and constitutes a major polypeptide in neural scars (Goldman et al., 1978). Because these astroglial scars appear to be involved in preventing brain regeneration and recovery of function, an understanding of the factors governing the polymerization of GFAP may be of both basic and clinical interest (Brock & O'Callaghan, 1987). This paper describes the effects of some cations, nucleotides, amino acids, and dephosphorylation on GFAP assembly and discusses the involvement of GFAP domains in assembly.

Materials and Methods

Materials. Bovine α-chymotrypsin (EC 3.4.21.1), alkaline phosphatase (EC 3.1.3.1) from human placenta (17 units/mg), iodoacetamide, and PMSF were obtained from Sigma (St. Louis, MO). BNPS-skatole was from Pierce (Rockford, IL). All other reagents were of analytical grade.

GFAP Preparation. GFAP was prepared from bovine spinal cords according to Tokutake et al. (1983) with slight modifications. Iodoacetamide (0.5 mM) was substituted for PMSF throughout the procedure. GFAP was separated from the detergent-insoluble IF pellets by hydroxylapatite chromatography in the presence of 8 M urea. GFAP subfractions were dialyzed at 4 °C overnight with one change against 2 mM Tris-HCl, pH 7.5, containing 0.5 mM iodoacetamide (disassembly buffer). Insoluble materials were removed by centrifugation at 17000*g* for 30 min. The supernatant was dialyzed at 4 °C against 100 mM imidazole hydrochloride, pH 6.8, containing 0.5 mM iodoacetamide (assembly buffer). The reassembled filaments were collected by centrifugation at 70000*g* for 60 min. The resulting pellets were dissolved in 8 M urea made up in 2 mM Tris-HCl, pH 7.5, and stored at 4 °C (GFAP stock solution). Urea was removed from this GFAP solution before use by overnight dialysis at 4 °C against 2 mM Tris-HCl, pH 6.8–7.5. Before polymerization experiments GFAP solutions were centrifuged at 70000*g* for 30 min to remove aggregates.

Limited Chymotryptic Digestion and GFAP Reassembly. GFAP dissolved in 2 mM Tris-HCl, pH 7.5, was incubated at room temperature with α-chymotrypsin at a substrate to enzyme ratio of 200:1 or 20:1 (w/w). Digestion was terminated by addition of PMSF to a final concentration of 0.2 mM. GFAP assembly was promoted by addition of concentrated imidazole hydrochloride, pH 6.8, to 100 mM. At 30 min, aliquots were taken for electron microscopy, and after incubation for periods ranging from 1 h to overnight, the resulting filaments were collected by centrifugation. Polypeptides in the supernatant were precipitated by 5% (w/v) trichloroacetic acid. The polypeptide composition was analyzed by SDS gel electrophoresis (Laemmli, 1970) as modified by De Blas et al. (1979).

GFAP Cleavage with BNPS-skatole. GFAP stock solution was mixed with an equal volume of BNPS-skatole solution made up in glacial acetic acid with a protein to reagent ratio of 3:1 (w/w). This mixture was stirred at room temperature for 2 days. Excess BNPS-skatole was removed by extensive dialysis against 50% acetic acid. Polypeptides were precipitated by 5% (w/v) trichloroacetic acid and redissolved in 8 M urea made up in 2 mM Tris-HCl buffer, pH 7.5. Insoluble materials were removed by centrifugation at 17000*g* for 30 min. Urea was removed by overnight dialysis against 1000 volumes of 2 mM Tris-HCl, pH 7.5. After introduction of the assembly buffer, this solution was incubated at room temperature overnight. After centrifugation at 70000*g* for 30 min, the pellet was collected, and polypeptides in the supernatant were isolated by trichloroacetic acid precipitation. The polypeptide composition in both fractions was analyzed by SDS gel electrophoresis.

Measurement of GFAP Reassembly. Soluble GFAP in disassembly buffer, pH 6.9, was assembled into filaments upon addition of various reagents. This process was accompanied by development of turbidity which was measured spectrophotometrically at 300 nm. When the effects of nucleoside triphosphates on filament formation were measured, turbidity was monitored at 320 nm to eliminate interference by purine or pyrimidine ring absorbance.

GFAP Dephosphorylation and GFAP Reassembly. GFAP solution, made up in disassembly buffer, pH 8.0, was incubated at 37 °C for 4 h with alkaline phosphatase (0.6 enzyme unit/mg of GFAP). The same amount of boiled enzyme was included in control incubations. Then concentrated imidazole hydrochloride buffer, pH 6.8, was added to 100 mM, and turbidity was measured 1 h later. GFAP was precipitated with 5% (w/v) trichloroacetic acid, and the phosphate released

[1] Abbreviations: GFAP, glial fibrillary acidic protein; IF, intermediate filament; NF neurofilament(s); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

FIGURE 1: Purified glial fibrillary acidic protein. GFAP was electrophoresed on 12.8% SDS–polyacrylamide gels as described under Materials and Methods.

during dephosphorylation was measured as described by Bechtel et al. (1977). Polypeptides were analyzed electrophoretically on SDS–polyacrylamide gels.

Protein Determinations. The Bradford protein assay (Bradford, 1976) was used for determination of GFAP concentrations.

Electron Microscopy. After polymerization of 1 mg/mL GFAP for 30 min at room temperature drops of the filament suspension were placed on carbon-coated grids for electron microscopy. After 60 s the drops were removed by touching with filter paper. Then the grids were stained with 1% uranyl acetate for 30 s. Excess uranyl acetate was removed with filter paper, and the grids were air-dried.

RESULTS

GFAP, isolated from bovine spinal cords and purified by hydroxylapatite chromatography and a cycle of disassembly and reassembly, was a single polypeptide with a molecular mass of 50 000 daltons. It was susceptible to proteases, but inclusion of a sulfhydryl reagent, iodoacetamide, throughout preparation largely inhibited proteolysis so that only a small amount of a GFAP fragment with a slightly lower molecular weight could be detected (Figure 1).

GFAP could be stored in 8 M urea at 4 °C for up to 1 month without detectable changes when subjected to SDS–polyacrylamide gel electrophoresis. When GFAP stock solution was dialyzed into low ionic strength solution, 2 mM Tris-HCl, pH 7.5, it remained soluble. Electron microscopy (Figure 2) revealed that the GFAP consisted of protomers and small aggregates. To remove aggregates, GFAP solutions were routinely centrifuged at 70000*g* for 30 min before polymerization.

GFAP was incubated with increasing concentrations of cations at room temperature. Turbidity measurements on the opalescent suspensions showed that low concentrations of divalent cations promoted GFAP assembly. Electron microscopy (Figure 3) demonstrated that 1 mg/mL GFAP in 60 mM KCl and 5 mM $CaCl_2$ assembled after 30 min into intermediate filaments which usually associated through side-by-side interactions to form striated filament bundles, though individual filaments still could be discerned (arrows).

The chlorides of calcium, magnesium, and barium showed similar abilities to promote GFAP assembly (Figure 4A). At divalent cation concentrations above 10 mM the turbidity increases leveled off. Further increases in divalent cation concentrations induced rapid dropping in apparent turbidity (data not shown). This drop was caused by rapid GFAP aggregation, formation of white flocculent material, and precipitation. Copper chloride, even in the micromolar range,

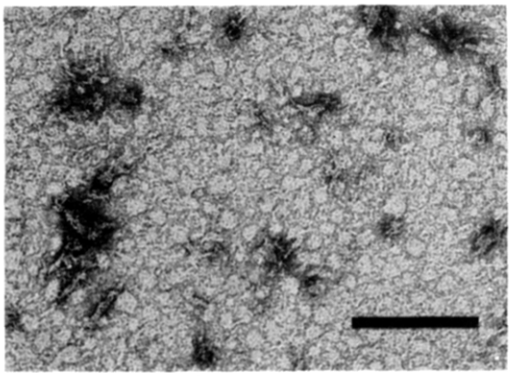

FIGURE 2: Electron micrograph of a negatively stained preparation of depolymerized glial fibrillary acidic protein (GFAP). GFAP (1.0 mg/mL) in 8 M urea was dialyzed against 2 mM Tris-HCl, pH 7.5. Drops of this solution were applied to carbon-coated electron microscopy grids and stained with 1% uranyl acetate. The bar represents 200 nm.

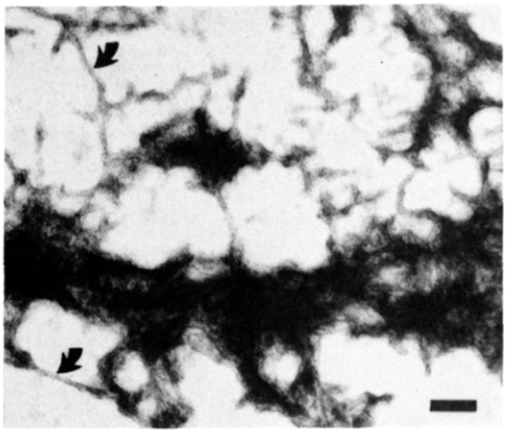

FIGURE 3: Electron micrograph of a negatively stained preparation of polymerized GFAP. GFAP (1.0 mg/mL) in 2 mM Tris-HCl, pH 7.5, was centrifuged for 30 min at 70000*g*. GFAP in the supernatant was polymerized by additions of concentrated KCl and $CaCl_2$ to 60 mM and 5 mM, respectively. Then GFAP solutions were kept at room temperature for 30 min before application to the electron microscopy grids and preparation as described under Materials and Methods. The bar represents 200 nm, and arrows point to some individual filaments.

rapidly induced precipitation (data not shown), so the effects of copper and manganese were studied by monitoring turbidity development in the initial few minutes (Figure 4B). Heavy aggregate was rapidly formed upon the addition of copper chloride as low as 10 μM, and its turbidity at 300 nm reached a plateau in 5 min. At the same concentration manganese chloride and calcium chloride barely initiated turbidity development though manganese ion showed a stronger effect than calcium.

Monovalent cations promoted GFAP assembly much less effectively than did divalent cations (Figure 5). Sodium demonstrated almost the same effect on GFAP assembly as potassium while lithium showed the strongest effect among these three cations. Higher levels of assembly were observed for increasing cation concentrations with a maximum at about 200 mM. At higher cation concentrations turbidity decreased. Though the strength of their effects might be different, the overall effects of these three monovalent cations were rather similar.

Turbidity of GFAP solutions made up in 2 mM Tris-HCl, pH 6.9, containing 60 mM KCl and 1 mM $CaCl_2$ was directly proportional to the GFAP concentration up to 1.4 mg of GFAP/mL. The data gave a critical protein concentration of 0.08 ± 0.04 mg of GFAP/mL, below which no turbidity

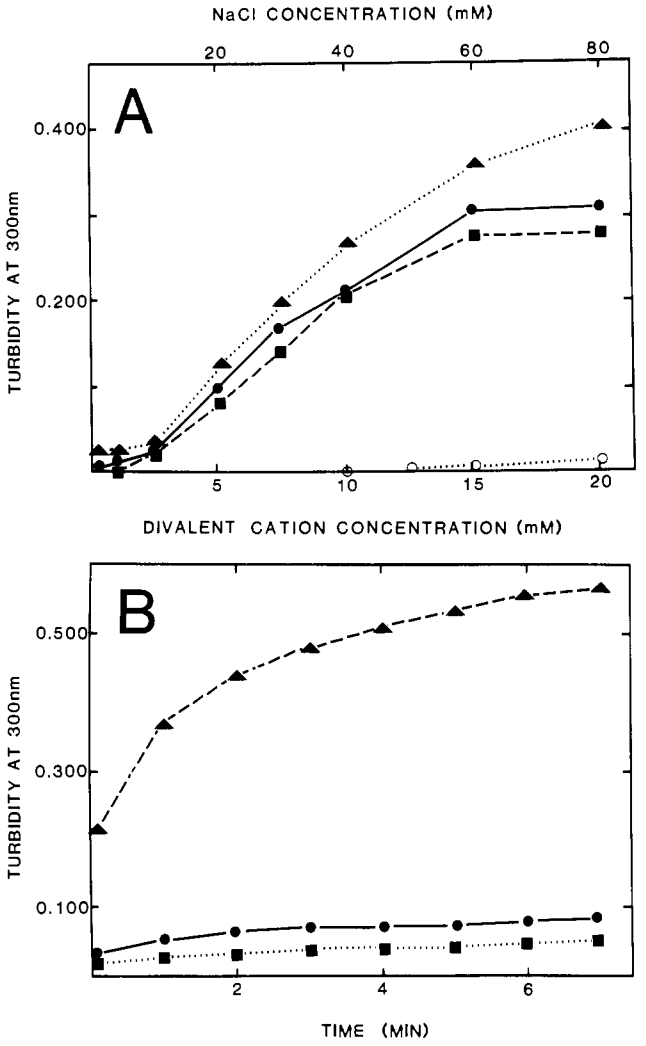

FIGURE 4: Effects of divalent cations on GFAP assembly. GFAP stock solution was dialyzed overnight at 4 °C against disassembly buffer, pH 6.9. Various ions were added, and solutions were incubated for 2 h at room temperature. Then turbidity was measured at 300 nm. (A) Ions studied were calcium chloride (▲), barium chloride (■), or magnesium chloride (●). For comparison, the turbidity developed by sodium chloride (○) also was plotted. Potassium chloride (60 mM) was included in all samples. (B) Copper chloride (▲) and manganese chloride (●) were separately added to a final concentration of 10 μM to 0.26 mg/mL GFAP solution prepared as described in Figure 2. The turbidity was measured at 1-min intervals. Potassium chloride (60 mM) was included. For comparison, turbidity developed by calcium chloride (■) at the same concentration also was plotted.

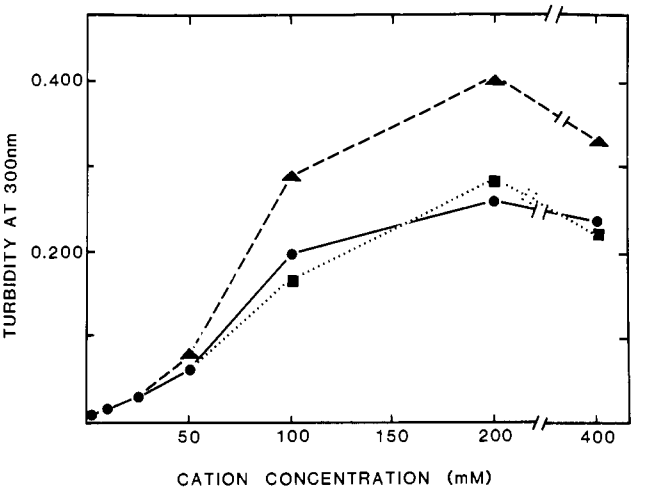

FIGURE 5: Effects of monovalent cations on GFAP assembly. GFAP stock solution was diluted and dialyzed overnight at 4 °C against disassembly buffer, pH 6.9. Potassium chloride (■), sodium chloride (●), and lithium chloride (▲) were separately added to GFAP solutions (0.26 mg of protein/mL). Mixtures were incubated at room temperature for 2 h, and turbidity at 300 nm was measured.

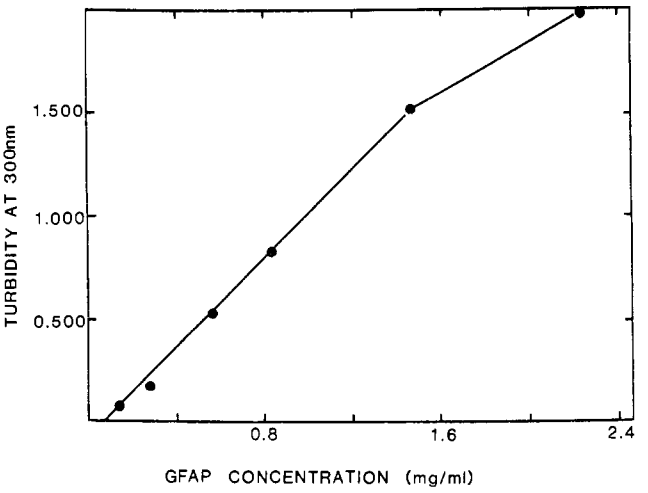

FIGURE 6: Relationship between GFAP concentration and turbidity. Turbidity was developed in GFAP solutions of various concentration upon addition of 1 mM $CaCl_2$ and 60 mM KCl. After 2 h of assembly at room temperature, the turbidity was measured at 300 nm.

increase could be detected (Figure 6).

When subjected to chymotryptic digestion at a substrate to enzyme ratio of 200:1 (w/w), a predominant M_r 38 000 rod domain and a number of less digested GFAP derivatives were observed on SDS–polyacrylamide gels (Figure 7A, lane 1). After the addition of 100 mM imidazole buffer, pH 6.8, turbidity gradually developed. The polymer produced by overnight incubation at room temperature was collected by centrifugation at 70000*g* for 30 min. The pellet (Figure 7A, lane 2) contained a higher ratio of less digested polypeptides and less M_r 38 000 rod domain, compared with polypeptides in the supernatant (Figure 7A, lane 3). When the GFAP to chymotrypsin ratio was increased to 20:1 (w/w), the relatively stable rod domain was obtained exclusively (Figure 7A, lane 4), and a 10-min digestion severely reduced GFAP's ability to develop turbidity. Centrifugation at 70000*g* for 30 min produced little if any pellet from the intensively digested GFAP when assembly was initiated 1 h before by the addition of 100 mM imidazole buffer although the centrifugal force used was strong enough to pellet the assembled filaments from undigested GFAP. This indicated that removal of the end domains increased GFAP solubility in assembly buffer and greatly reduced its ability to form filaments.

GFAP assembled after chymotrypsin digestion was examined by electron microscopy (Figure 8). GFAP digested at a ratio of 200:1 to chymotrypsin revealed intermediate filaments which did not assemble into bundles despite the GFAP being rather concentrated initially (1 mg/mL). After digestion of GFAP with a higher level (20:1) of chymotrypsin, material was produced which did not assemble into morphologically recognizable filaments, and only small amounts of protein (possibly aggregates) could be pelleted by centrifugation (data not shown). The chymotrypsin digestion experiments showed, therefore, that removal of GFAP head and tail pieces decreased the polymerization ability of GFAP while eliminating the ability of GFAP filaments to assemble into bundles through side-by-side associations.

GFAP was chemically cleaved with BNPS-skatole to further study the role of the end domains in assembly. Fortunately cow GFAP, like mouse GFAP, appeared to contain only one

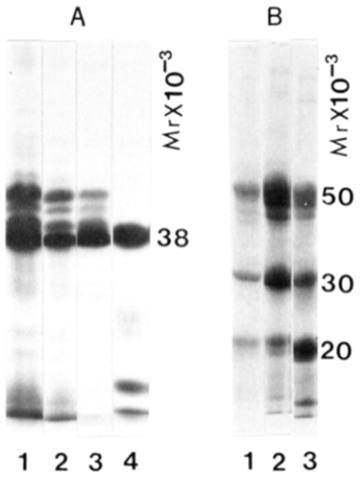

FIGURE 7: SDS gel electrophoresis of soluble and coassembled GFAP peptides. (A) GFAP was treated with α-chymotrypsin at a GFAP to enzyme ratio of 200:1 for 10 min as described under Materials and Methods. With incubation at room temperature overnight in assembly buffer, turbidity developed in the fragmented GFAP solution. The assembled GFAP was collected by centrifugation at 70000*g* for 30 min, and polypeptides in the supernatant were precipitated by adding trichloroacetic acid to 5% (w/v). (Lane 1) Chymotryptic digest of GFAP; (lane 2) reassembled chymotrypsin-treated GFAP pellet; (lane 3) supernatant of chymotrypsin-treated GFAP after assembly and centrifugation; (lane 4) GFAP rod domain obtained by chymotryptic treatment at a GFAP to enzyme ratio of 20:1. (B) GFAP was cleaved by BNPS-skatole treatment as described under Materials and Methods. After addition of assembly buffer turbidity formed, and assembled GFAP was separated from soluble peptides as described in (A). (Lane 1) BNPS-skatole-cleaved GFAP; (lane 2) assembled GFAP pellet; (lane 3) supernatant.

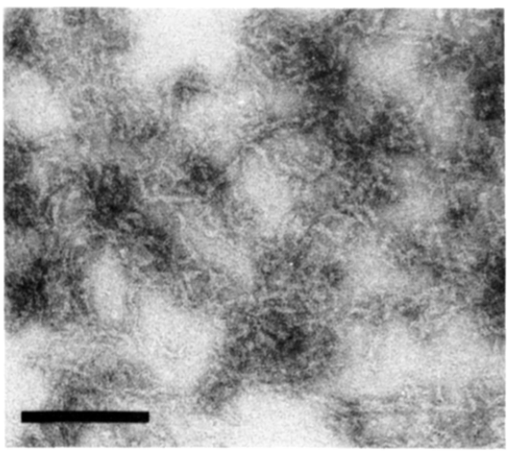

FIGURE 8: Electron micrograph of a negatively stained preparation of GFAP polymerized after chymotrypsin digestion. GFAP was digested with chymotrypsin at a protein to enzyme ratio of 200:1 (w/w) for 10 min before polymerization by the addition of concentrated PMSF to 0.2 mM and imidazole hydrochloride, pH 6.8, to 100 mM. After 30 min at room temperature, aliquots were examined by electron microscopy. Bar represents 200 nm.

tryptophan (Lewis et al., 1984) which could be cleaved specifically by BNPS-skatole. Two fragments, the N-terminal M_r 30000 polypeptide and the C-terminal M_r 20000 polypeptide, were produced together with some intact GFAP molecules (Figure 7B, lane 1). Turbidity slowly developed after addition of assembly buffer to the solution of these polypeptides. Separated by centrifugation at 70000*g* for 30 min, the polypeptides in the pellet and in the supernatant were analyzed by SDS gel electrophoresis (Figure 7B, lanes 2 and 3). All three polypeptides were found in both supernatant and pellet, but in different abundance; more M_r 30000 polypeptide coassembled with intact GFAP (Figure 7B, lane 2) while the

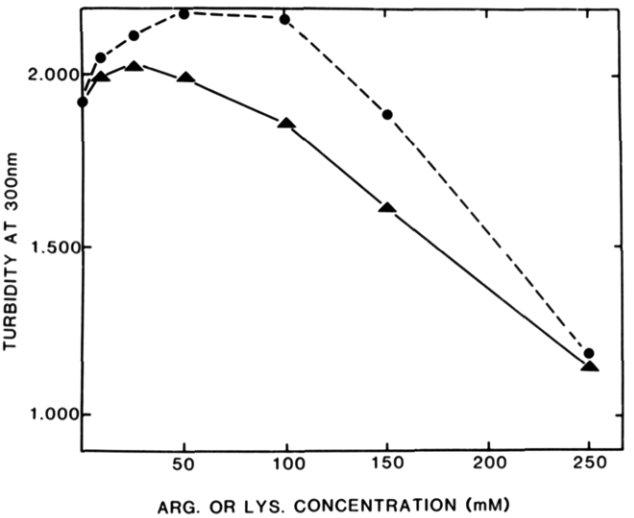

FIGURE 9: Effects of arginine and lysine on GFAP assembly. Soluble GFAP was prepared as described in Figure 4. Arginine (●) or lysine (▲) at various concentrations was added to assembly buffer. Turbidity was measured at 300 nm after the GFAP solution (0.37 mg/mL) was incubated at room temperature overnight.

M_r 20000 polypeptide was predominantly in the supernatant (Figure 7B, lane 3). This indicated that the N-terminal M_r 30000 polypeptide had higher affinity for intact GFAP than did the M_r 20000 C-terminus.

The head domains of most IF proteins were found to be rich in arginine residues (Geisler et al., 1982, 1983a). Filament formation by vimentin and desmin was inhibited by 150 mM arginine (Traub & Vorgias, 1983). To test if this also was the case for GFAP, arginine or lysine at various concentrations was included in the assembly buffer (Figure 9). Data showed that below 100 mM arginine slightly promoted GFAP assembly. Lysine produced similar results though it was somewhat less effective. At concentrations above about 100 mM arginine and lysine inhibited filament formation.

To study the effects of nucleoside triphosphates on GFAP assembly, ATP, GTP, CTP, and UTP were separately added to a GFAP solution to final concentrations of 0.25–5.0 mM. Turbidity was measured at 320 nm 1 h after addition of assembly buffer. None of these nucleoside triphosphates showed significant promotion of GFAP assembly in this concentration range. Instead, at 5 mM they slightly diminished assembly (data not shown). In the absence of assembly buffer the same results were obtained.

GFAP is a phosphoprotein (Browning & Ruina, 1984; McCarthy et al., 1985). Treatment of GFAP with alkaline phosphatase (0.6 unit/mg of GFAP) at 37 °C for 4 h released approximately two to three phosphate groups per GFAP molecule. This treatment decreased the development of turbidity by about 20% but did not change gel patterns of the GFAP (not shown). These data suggested a small correlation between GFAP phosphorylation and polymerizability. We also noticed that storage of the GFAP stock solution resulted in a gradual decrease of phosphate content and a concomitant loss of polymerizability (data not shown).

Discussion

GFAP is the major constituent of astroglial intermediate filaments. It is extremely susceptible to proteases which play an important role in the metabolism of intermediate filaments. This proteolysis is triggered by increases in intracellular calcium concentration (Zimmerman & Schlaepfer, 1982) and involves preferential attack on the head region (Fischer et al., 1986). Further studies found that the calcium-activated

protease is a thiol protease (Zimmerman & Schlaepfer, 1984) which is very sensitive to sulfhydryl reagents. On the basis of this consideration iodoacetamide, one of the sulfhydryl reagents, was substituted for PMSF which was more effective for inhibiting serine proteases.

Rueger et al. (1979) reported that optimal conditions for GFAP assembly were 100 mM imidazole hydrochloride buffer, pH 6.8. Lower pH favored assembly. Under this condition GFAP filaments were formed within 30 min. The degree of assembly could be demonstrated by measuring the amount of protein in both the pellet and the supernatant separated by centrifugation or by the spectrophotometric measurement of solution turbidity. Zackroff and Goldman (1979) demonstrated that the amount of IF found in the pellet was directly proportional to solution optical density at 300 nm. On the basis of these observations the turbidity of a GFAP solution at 300 or 320 nm was used to characterize its state of assembly in this paper.

The soluble GFAP, like most other IF counterparts, readily reassembled into filaments in higher ionic strength solutions (Steinert et al., 1976, 1981; Rueger et al., 1979; Huiatt et al., 1980) so that cycles of assembly and disassembly through solution ionic strength adjustments could be used as part of the purification scheme. Our data showed maximal turbidity increases in about 200 mM monovalent cation solutions (Figure 5). This result demonstrated that physiological conditions favored the stability of GFAP filaments. Contrary to the general understanding that IFs were insoluble in high ionic strength solutions, at around 400 mM monovalent cation GFAP was somewhat more soluble. This implied that increased ionic strength probably interrupted intermolecular attractions and, as a consequence, caused the increase in protein solubility. Zackroff and Goldman (1980) described extraction of crude IFs from squid brain with a buffer containing 1 M KCl. When this solution was diluted 10 times, IFs were reassembled.

Divalent cations promoted GFAP assembly much more effectively than monovalent cations (Figure 4A). This indicated that divalent cations might promote assembly through a different mechanism. Our experimental data suggested that divalent cations might have one or more specific binding site(s) on GFAP. Potassium chloride at 60 mM did not eliminate the binding of $^{45}Ca^{2+}$ at approximately 1 μM, implying that potassium probably did not compete for the calcium binding site (Yang et al., 1988). Mg^{2+}, Ca^{2+}, and Ba^{2+} demonstrated very comparable effects on GFAP assembly (Figure 4A). Cu^{2+} showed an extreme ability to induce GFAP aggregation (Figure 4b) indicated by formation of a white, flocculent precipitate rather than the opalescent turbidity observed in a filament suspension. A similar effect of Cu^{2+} on keratin was described by Fukuyama et al. (1978). The turbidity increases produced by Mn^{2+}, as well as its location in the periodic table, were between those of copper and calcium. Our results are quite similar to the observations of monovalent and divalent cation effects on desmin filament assembly reported by Stromer et al. (1987). They too found that divalent cations were much more effective than monovalent cations at promoting intermediate filament assembly.

Rueger et al. (1979) detected filament formation at as low as 0.01 mg of protein/mL, lower than our result of 0.08 ± 0.04 mg of protein/mL, probably due to different assembling conditions. Apparently their 100 mM imidazole buffer was more capable of initiating assembly than the 1 mM $CaCl_2$ and 60 mM KCl used in our experiments. The difference of assembly buffers also may have produced a difference in the appearance of the resulting filaments: more individual filaments and less bundles were described by Rueger et al. However, a more likely explanation relates to the fact that the GFAP solutions used in our experiments were 10-fold more concentrated than were the GFAP solutions polymerized by Rueger et al. (1 mg/mL vs 0.1 mg/mL). High GFAP concentrations would promote the formation of higher order structures after filament formation (Figure 3). The diameters of these filaments (15–16 nm) agree well with the results of Stromer et al. (1987) that desmin filament diameters were decreased with time of polymerization and increased with polymerization temperature and the presence of divalent cations, particularly calcium, which yielded the thickest filaments they detected.

IF proteins consist of three domains: a basic headpiece, a M_r 38 000 rod domain, and a tailpiece of variable size (Geisler & Weber, 1982). Limited chymotryptic cleavage removes the two end domains and leaves the rod domain intact due to its chymotryptic resistance. As a member of the IF family, GFAP can yield this M_r 38 000 rod domain in the same way (Rueger et al., 1981). Under conditions favoring IF assembly the rod domains obtained from chymotrypsin-treated intermediate filament proteins do not assemble to the same extent as undigested proteins (Figure 8). When some intact GFAP remains mixed with the rods, apparently normal filaments assemble, but they do not form bundles (Figure 8). When chymotrypsin digestion is complete and only the rod remains, morphologically recognizable intermediate filaments cannot be detected. In this regard our results with GFAP are quite similar to those of other workers studying polymerization of other intermediate filament proteins, e.g., Geisler et al. (1982) and Traub and Vorgias (1983). Geisler et al. could form ribbons from the rod domain but only at low pH (pH 5.5). This led to the hypothesis that the nonhelical headpiece appeared to play a role both in linear alignment and in lateral interactions which were important for packing the subunits into 7–10-nm filaments (Geisler et al., 1982; Geisler & Weber, 1982; Steinert et al., 1983). According to this hypothesis, the headpiece must have a high affinity for the intact subunits. Our experiments supported this hypothesis by demonstrating that under assembling conditions more N-terminal M_r 30 000 fragment was found to coassemble with intact GFAP than the C-terminal M_r 20 000 polypeptide, which was predominant in the supernatant (Figure 7B). In contrast, the C-terminal of intermediate filament proteins appears to contribute little to filament formation (Kaufmann et al., 1985).

Arginine residues on the headpiece were reported to play an important role in forming filaments by interacting with acidic residues on the rod domain of adjacent IF molecules. Inclusion of 150 mM arginine in the assembly buffer inhibited vimentin and desmin assembly (Traub & Vorgias, 1983). Our data showed that inclusion of arginine or lysine at high concentrations—above about 100 mM—inhibited GFAP assembly though lysine was reported not to inhibit desmin and vimentin assembly (Traub & Vorgias, 1983). Structurally, the headpiece of GFAP is different from that of desmin and vimentin (Lewis et al., 1984). First, GFAP has a much shorter headpiece which consists of approximately 40 amino acid residues. Second, in the headpiece there are only five arginine residues and three acidic residues compared with ten arginines and no acidic residues for either desmin or vimentin. On the basis of these facts, it is understandable that arginine residues showed less specific involvement in GFAP filament formation.

GFAP is a phosphoprotein (Browning & Ruina, 1984; McCarthy et al., 1985). In 1984 Wong et al. reported that

the phosphorylation state affected association of the M_r 150000 neurofilament protein with the filament. Georges et al. (1986) argued that dephosphorylation of neurofilaments had no effect on reassembly of subunits. Dephosphorylated neurofilament subunits certainly were assembly competent. However, when equal amounts of native and dephosphorylated NF subunits were mixed together, more native M_r 150000 and M_r 200000 subunits were assembled into filaments than their dephosphorylated forms, as also shown in the paper of Georges et al. Our data on GFAP demonstrated that dephosphorylation of GFAP subunits slightly reduced assembling ability but did not abolish it. This resolved the apparent contradiction between the conclusions of Wong et al. (1984) and Georges et al. (1986). Most of the phosphorylation sites in NF are peripherally located (Julien & Mushynski, 1983). In GFAP, they probably are located mainly on the tailpiece which is rich in serine and threonine residues, several of which may occur in consensus phosphorylation sequences (Geisler et al., 1982). Although the tailpiece is not vital for IF assembly (Kaufmann et al., 1985), a conformational change of this domain induced by dephosphorylation appears to affect turbidity. The tailpiece is proposed to communicate between filaments and the cytoplasm (Geisler & Weber, 1986), and phosphorylation may regulate the packing of individual filaments into bundles and/or mediate developmental effects (Dahl et al., 1986) or interactions between IFs and other filaments or organelles. Such interactions between filaments would not change turbidity as much as filament formation itself. On the other hand, phosphorylation of vimentin (Inagaki et al., 1987) and desmin (Geisler & Weber, 1988) by protein kinase A inhibits filament formation. The phosphorylation sites in desmin have been localized to the head region (Geisler & Weber, 1988) so it may be that different protein kinases can phosphorylate different regions of the intermediate filament proteins to modulate a variety of functions.

ATP is involved in the formation of microfilaments (Korn, 1982), and GTP plays a similar role in the formation of microtubules (Weisenberg et al., 1968; Gaskin, 1981). We wondered if one of these two or another nucleoside triphosphate was involved in the formation of GFAP filaments, but this did not appear to be the case. Our observations indicated that nucleoside triphosphates are not necessary for GFAP assembly. This observation agreed with that of Zackroff et al. (1982), who reported ATP and GTP at 1–5 mM did not affect IF assembly. High concentrations of nucleoside triphosphates slightly inhibited assembly due perhaps to the introduction to the assembly buffer of negative charge. We noticed that, without carefully bringing the pH of the nucleoside triphosphate solution to 6.8–7.0, addition of this solution into a GFAP solution made up in 2 mM Tris-HCl, pH 6.9, would promote rapid assembly due to acidification of the GFAP solution.

To sum up, our data showed that a number of factors can modulate GFAP assembly. Divalent cations are much stronger in accelerating assembly than monovalent cations, possibly due to the existence on GFAP of specific divalent cation binding sites (see next paper). The rod domain of GFAP is more soluble than the intact molecule. The shorter and less basic GFAP headpiece appears to play a less important role in assembly than its other IF counterparts, but more investigation needs to be done to explore the nature of the higher affinity of the N-terminal M_r 30000 fragment for the intact molecule. Dephosphorylation slightly inhibits GFAP assembly, but phosphorylation of different regions may modulate different GFAP functions.

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Registry No. K, 7440-09-7; Na, 7440-23-5; Li, 7439-93-2; Ca, 7440-70-2; Mg, 7439-95-4; Mn, 7439-96-5; Ba, 7440-39-3; Cu, 7440-50-8; L-arginine, 74-79-3; L-lysine, 56-87-1.

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Characterization and Location of Divalent Cation Binding Sites in Bovine Glial Fibrillary Acidic Protein†

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ABSTRACT: In our previous work [Yang, Z. W., & Babitch, J. A. (1988) *Biochemistry* (preceding paper in this issue)] divalent cations were found to be more effective promoters of astroglial filament formation than were monovalent cations. To determine if one or more divalent cation binding sites were the basis for this difference, glial fibrillary acidic protein (GFAP) was attached to nitrocellulose membranes and bathed in 1 μM $^{45}CaCl_2$ in 60 mM KCl, 0.5 mM $MgCl_2$, and 10 mM imidazole hydrochloride, pH 7.4. After removal of unbound $^{45}Ca^{2+}$, GFAP was observed to bind calcium. Flow dialysis experiments showed that GFAP, dissolved in 2 mM Tris-HCl, pH 7.5, contained three classes of binding sites and 0.61 ± 0.08 (SD), 1.7 ± 0.4, and 4.6 ± 0.2 sites per GFAP molecule with dissociation constants of 0.66 ± 0.01 μM, 6.6 ± 0.3 μM, and 44 ± 1 μM, respectively. After addition of 0.5 mM $MgSO_4$ to the flow dialysis solution, the high- and low-affinity sites were not observed while the remaining sites (1.95 ± 0.15 per GFAP molecule) had a K_d = 2.16 ± 0.25 μM. This showed that the high- and low-affinity sites are "Ca^{2+}–Mg^{2+}" sites while sites with intermediate affinity are calcium specific. To locate the calcium-binding regions, GFAP peptides were examined for calcium binding by calcium-45 autoradiography. The calcium-specific binding areas were localized in coil I. Computer-assisted analysis of the GFAP sequence revealed several EF-hand-like areas which could be the calcium binding sites. We conclude that divalent cations may play both structural and regulatory roles in astroglial intermediate filaments.

Intermediate filament proteins consist of a predominantly helical rod domain and two flanking end pieces (Geisler et al., 1982, 1983). In low ionic strength solutions, intermediate filaments are soluble, but increases in ionic strength promote filament assembly (Steinert et al., 1976, 1981; Rueger et al., 1979; Huiatt et al., 1980; Yang & Babitch, 1988). Divalent cations may act through different mechanisms from monovalent cations in accelerating filament formation (Fukuyama et al., 1978).

Glial fibrillary acidic protein (GFAP)[1] is an intermediate

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