Molecular Aging of Tubulin: Accumulation of Isoaspartyl Sites in Vitro and in Vivo†

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ABSTRACT: The formation of isoaspartyl sites during aging of rat tubulin in vitro and in vivo has been studied. When incubated in vitro at pH 7.4, 37 °C, purified rat brain tubulin accumulated isoaspartyl sites at a rate ≥2.4 isoaspartyl sites per 100 tubulin subunits (50 kDa) per day for 30 days. Isoaspartate levels were estimated by the transfer of radiolabeled methyl groups from *S*-adenosyl-L-[*methyl*-^{3}H]-methionine in a reaction catalyzed by protein-L-isoaspartyl methyltransferase. Isoaspartate formation occurred in parallel with, but was not dependent upon, extensive cross-linking of tubulin via formation of intermolecular disulfide bonds. When rat PC12 cells were incubated for 24 or 72 h in the presence of adenosine dialdehyde, a potent methyltransferase inhibitor, a substantial and consistent increase in the isoaspartate content of tubulin was observed. This suggests that tubulin constantly undergoes isoaspartate formation in vivo, but that the levels are normally kept low by methylation-dependent repair. These findings support the hypothesis that protein-L-isoaspartyl methyltransferase plays a key role in countering spontaneous damage reactions to proteins associated with cell aging. These results also suggest that tubulin is an important target for protein-L-isoaspartyl methyltransferase in vivo.

Protein-L-isoaspartyl methyltransferase (PIMT),[1] EC 2.1.1.77, is a widely distributed enzyme that catalyzes transfer of the active methyl group of *S*-adenosyl-L-methionine (AdoMet) onto the α-carboxyl group of atypical L-isoaspartyl sites in peptides and proteins [reviewed by Aswad (1995) and by Lowenson and Clarke (1995)]. Isoaspartyl sites are formed by spontaneous intramolecular deamidation at Asn-X linkages or by isomerization of Asp-X linkages, resulting in a peptide bond that occurs through the side chain *β*-carbonyl of Asp (Figure 1). This reaction has been observed most often at Asn-Gly, Asn-Ser, and Asp-Gly sequences when they occur in flexible peptides or in flexible domains of proteins (Brennan & Clarke, 1995; Johnson & Aswad, 1995; Wright, 1995). Isoaspartate formation is a common mechanism for protein damage under mild conditions in vitro and may constitute a major pathway of protein aging and damage in vivo.

There is mounting evidence that PIMT functions in cells to repair atypical isoaspartate-bearing proteins. When PIMT methylates the free α-carboxyl group at isoaspartyl sites in model peptides, the resulting methyl ester rapidly decomposes to the succinimide, which in turn hydrolyzes to form a mixture of isoaspartyl and aspartyl peptides (Figure 2). Each cycle of methylation/demethylation thus "repairs" a portion (typically 15−35%) of the isoaspartyl peptide bond by converting it to a normal aspartyl peptide. According to this mechanism, prolonged incubation of an isoaspartyl peptide with PIMT and excess AdoMet should lead to a net conversion of the isopeptide bond to a normal peptide bond. This prediction has been borne out with several model peptides (Galletti et al., 1988; Johnson et al., 1987b;

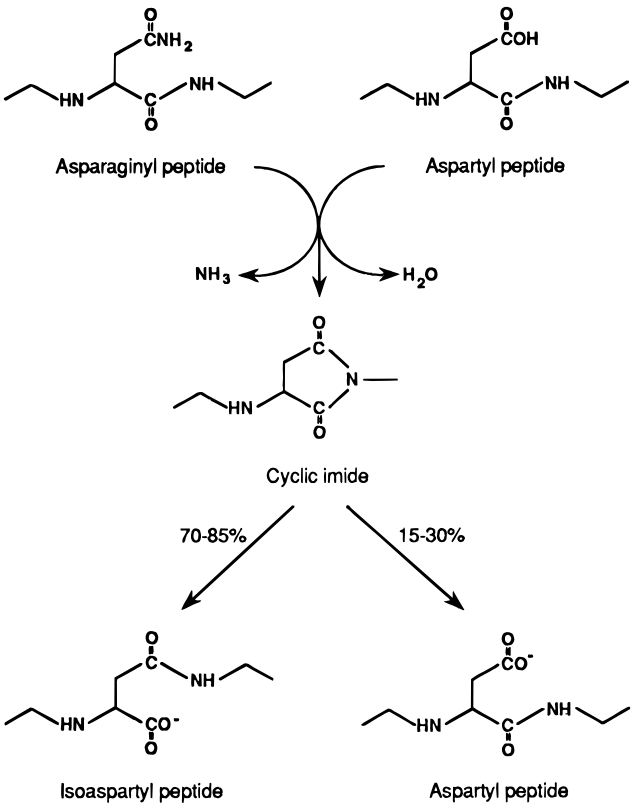

FIGURE 1: Mechanism of formation of L-isoaspartyl sites in proteins from L-asparaginyl or L-aspartyl sites. Based on Bornstein and Balian (1977).

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdOx, adenosine dialdehyde; 16-BAC, benzyldimethyl-*N*-hexadecylammonium chloride; CPC, cetylpyridinium chloride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PIMT, protein-L-isoaspartyl methyltransferase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

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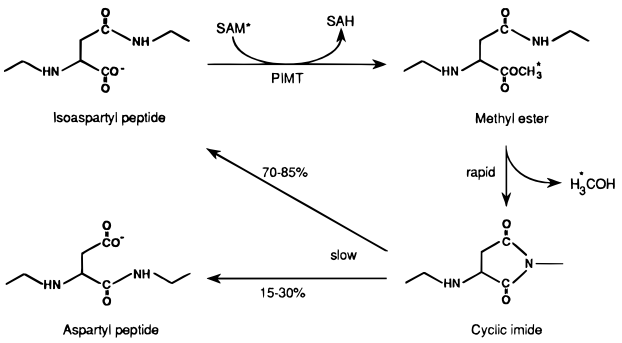

FIGURE 2: Mechanism for PIMT-dependent conversion of isoaspartyl sites to aspartyl sites. With model peptides, each cycle of enzymatic methylation and nonenzymatic demethylation results in the conversion of 15−30% of the atypical isoaspartyl peptide bonds to normal aspartyl peptide bonds. The overall conversion of the β-linkage to the α-linkage has been shown to occur with a half-life of about 8 h (Johnson et al., 1987b).

McFadden & Clarke, 1987). PIMT-dependent repair of age-damaged proteins has also been demonstrated. When bovine calmodulin was aged for 2 weeks at pH 7.4, 37 °C in the absence of Ca^{2+}, it generated multiple isoaspartyl sites in the calcium-binding domains (Potter et al., 1993), and its ability to activate Ca^{2+}/calmodulin-dependent protein kinase II dropped to only 18% of its normal value (Johnson et al., 1987a). After PIMT-dependent methylation for 48 h, the activity of the damaged calmodulin rose to 68% of normal. More recently, the partial repair of aged-damaged HPr phosphocarrier protein of *Escherichia coli* was demonstrated (Brennan et al., 1994). Functional repair of HPr was associated with an isoaspartate-to-aspartate conversion at the Asn^{12}-Gly^{13} position in HPr.

Recent studies with bacteria and mammalian cells further support a repair function for PIMT. Li and Clarke (1992) deleted the PIMT gene in *E. coli* and showed that, compared to the wild type, the mutant exhibited a significant decrease in survival during stationary phase and a decreased ability to survive heat shock. Isoaspartate would be expected to accumulate in proteins during stationary phase (when new protein synthesis is minimal) and in response to heat shock. The decreased viability of the mutant *E. coli* is consistent with a decreased ability to repair damaged key proteins. In a separate study, Johnson et al. (1993) cultured rat PC12 cells for 1−3 days in the presence of adenosine dialdehyde (AdOx), an inhibitor of *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase (Bartel & Borchardt, 1984; Hoffman, 1979). AdOx treatment causes a substantial rise in intracellular levels of AdoHcy, a strong competitive inhibitor of PIMT. As predicted by the repair hypothesis, AdOx treatment of PC12 cells for 3 days led to a substantial (5-fold or greater) increase in the methyl-accepting capacity of numerous cellular proteins. This was presumably due to the accumulation of unrepaired isoaspartyl sites.

The identification of in vivo substrates for PIMT may lead to important new insights on the role of protein damage and repair in cell aging. In a survey of endogenous substrates for PIMT in the cytosolic fraction of calf brain, our laboratory previously described a prominent methyl acceptor having an apparent molecular mass of 46 kDa as estimated by acidic PAGE in the presence of the cationic detergent cetylpyridinium chloride (CPC) (Aswad & Deight, 1983). Several years later, it was reported that tubulin is a major endogenous substrate for PIMT in the cytosol of both rat brain and calf brain (Ohta et al., 1987). In the latter study, tubulin (a 50 kDa protein) ran with an apparent molecular mass of 43 kDa in an acidic PAGE system that utilized the cationic detergent benzyldimethylcetylammonium chloride (16-BAC). The molecular mass discrepancy was attributed to the 16-BAC gel system. They suggested that the 46 kDa protein we had originally described was also tubulin.

The present study was designed to explore further the implication that tubulin is an important target for PIMT. We show first that in vitro aging of purified rat brain tubulin at pH 7.4, 37 °C leads to a substantial accumulation of isoaspartyl sites in the intact protein. We then utilize the AdOx strategy to demonstrate in cultured rat PC12 cells that tubulin accumulates isoaspartyl sites during methyltransferase inhibition, suggesting that it is also an in vivo substrate. Finally, we show that, although tubulin is a substrate for PIMT both in vitro and in vivo, it is distinct from the prominent methyl acceptor in rat brain cytosol that exhibits an apparent molecular mass of 46 kDa in acidic SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis).

EXPERIMENTAL PROCEDURES

Materials. Tubulin was purified from brain tissue of Sprague-Dawley rats (2−6 months of age) by the reversible assembly method of Williams and Lee (1982). The tubulin was found to be 95% pure by densitometer analysis after SDS−PAGE. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (15 Ci/mmol) was obtained from Dupont-NEN. The specific activity was adjusted by mixing with unlabeled AdoMet obtained from Sigma Chemical Co. Taxol was also obtained from Sigma.

The type I isoform of PIMT was purified from bovine brain as described previously (Henzel et al., 1989; Potter et al., 1992), and was homogeneous as judged by SDS−PAGE in 12% acrylamide. Enzyme prepared by this method has been shown previously to be free of detectable contaminants both by SDS−PAGE and by reversed-phase HPLC (Potter et al., 1992). Studies with a variety of synthetic peptides (Aswad & Johnson, 1987) and with tryptic digests of recombinant human growth hormone (Johnson et al., 1989b), bovine calmodulin (Potter et al., 1993), and recombinant human tissue plasminogen activator (Paranandi et al., 1994) demonstrate that bovine brain PIMT prepared in this manner methylates only L-isoaspartyl residues.

Preparation of Rat Brain Cytosol for Purification of Tubulin and for Analysis of Endogenous Methyl Acceptors. The following procedures were carried out at 2−6 °C. Whole brains (31 g) from Sprague-Dawley rats (2−6 months of age) were prehomogenized for 4 s in a Waring blender at low speed in the presence of 31 mL of 0.1 M sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Na-PIPES), pH 6.9, containing 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 4 M glycerol. Homogenization was completed by 6 passes in a Teflon−glass homogenizer with the pestle rotating at approximately 600 rpm. The homogenate was centrifuged at 6500*g* for 15 min. The supernatant was recovered and centrifuged at 96000*g* for 75 min. This supernatant, 16.5 mg/mL in protein, was recovered and stored at −70 °C prior to use.

Methylation of Proteins Prior to Gel Electrophoresis and Fluorography. Protein samples (6.7 μM purified tubulin,

3.6 mg/mL PC12 extracts, or 4.0 mg/mL rat brain cytosol) were methylated for 30 min at 30 °C in 50 mM K-MES, pH 6.2, containing 2.5 μM PIMT and 50 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (10−15 Ci/mmol). For methylation of PC12 proteins, the reactions also included 1 mM R3 peptide (Najbauer et al., 1993) to suppress arginine methylation. All reactions were stopped by the addition of SDS under conditions described in the individual figure legends.

Protein Determination. Protein was determined according to Lowry et al. (1951) after precipitation with 7% trichloroacetic acid.

Polyacrylamide Gel Electrophoresis and Fluorography. All protein electrophoresis was carried out in 0.75 mm thick gels of polyacrylamide using a Mini-Protean apparatus (BioRad Laboratories). The alkaline SDS−PAGE system used was that described by Laemmli and Favre (1973). Electrophoresis was carried out at ambient temperature and 120 V in a 10% resolving gel overlaid with a 4.8% stacking gel. This system uses a running buffer with a pH initially adjusted to 8.3; however, the pH in the resolving gel rises to a value of 9 or higher during a typical run. The pH 2.4 acidic SDS−PAGE system was that described by Fairbanks and Avruch (1972). Electrophoresis was carried out at ambient temperature and 50 V in a 10% resolving gel with no stacking gel.

Gels were stained with Coomassie Blue R-250, destained, equilibrated with water, and dried. For detection of tritium-labeled proteins by fluorography, gels were impregnated with sodium salicylate according to Chamberlain (1979) prior to drying. Fluorography was carried out at −70 °C with preflashed Kodak X-AR film as described by Laskey and Mills (1975).

Cell Culture and Preparation of Extracts. Rat PC12 cells were cultured in the presence or absence of 10 μM AdOx for 24 or 72 h (as indicated) and harvested as described previously (Najbauer & Aswad, 1990; Najbauer et al., 1993). Extracts were prepared by one of two methods, with all steps carried out at 0−4 °C. For method A (low-speed extract), approximately 5.2×10^8 cells were disrupted by sonication for 0.5 min in 4.0 mL of 10 mM sodium phosphate buffer, pH 7.2, containing 200 μM PMSF, 0.5 μg/mL leupeptin, and 1 mM EDTA. Sonicates were centrifuged at 38000*g* for 30 min. The supernatants, containing 5.3−6.6 mg/mL protein, were stored at −70 °C in the presence of 5% glycerol until use. For method B (high-speed extracts), approximately 4.6×10^8 cells were disrupted by sonication for 0.5 min in 3.3 mL of PPME buffer (100 mM Na-PIPES, pH 6.6, 200 μM PMSF, 0.5 μg/mL leupeptin, 1 mM $MgSO_4$, and 1 mM EGTA). Sonicates were centrifuged first at 48000*g* for 15 min. The resulting supernatants were then centrifuged at 180000*g* for 90 min to produce final extracts containing 6.3−7.5 mg/mL protein. These were stored at −70 °C in the presence of 5% glycerol until use.

Taxol-Promoted Tubulin Polymerization from Low-Speed and High-Speed Extracts of PC12 Cells. The low-speed extracts, prepared by method A above, were supplemented to contain PPME buffer (by addition of small volumes of the concentrated components) and then centrifuged at 48000*g* for 90 min at 0−4 °C. The supernatant was supplemented with an additional 1 mM $MgSO_4$ (final concentration), and then subjected to taxol-promoted polymerization according to Vallee (1986). Controls were included in which taxol was omitted during the polymerization steps. The pellets were resuspended in PCE methylation buffer (62.5 mM Na_2HPO_4, 95 mM citric acid buffer, pH 6.2, containing 2 mM EDTA), supplemented with 5% glycerol, and were stored frozen at −70 °C at a protein concentration of 1.0−2.1 mg/mL.

The high-speed extracts, prepared by method B above, were treated identically to the low-speed extracts with the following two exceptions: (1) the PPME addition was omitted since this extract was already in this buffer; (2) the polymerized microtubule pellets were washed twice in 100 mM Na-PIPES (pH 6.2) without disruption, prior to resuspension in the PCE buffer. The final preparation was stored at −70 °C in the presence of 5% glycerol at a protein concentration of 3.5 mg/mL.

RESULTS

Accumulation of Isoaspartyl Sites in Tubulin during in Vitro Aging. In vitro aging of purified proteins and peptides at pH 7.4, 37 °C has been used as a model for the accumulation of isoaspartyl sites under physiological conditions [reviewed by Johnson and Aswad, (1995); also see Sharma et al. (1993)]. The spontaneous rate of isoaspartate formation for structured proteins has been found to vary from near zero (cytochrome *c*, myoglobin, and lysozyme) to a high of approximately 8 mol % per day day for synapsin-1 (Johnson et al., 1989a; Paranandi & Aswad, 1995). If tubulin is a major substrate for PIMT in vivo, it should also exhibit significant isoaspartate formation during in vitro aging. To test this prediction, we incubated purified rat brain tubulin at pH 7.4, 37 °C for 30 days.[2] Samples were removed periodically during the incubation (every 2 days for the first 14 days, then every 4 days) and tested for their ability to accept methyl groups in the PIMT-catalyzed reaction. As shown in Figure 3, tubulin exhibited a nearly linear increase in methyl-accepting sites over the entire 30 day incubation, corresponding to an average rate of 2.4 mol % per day.[3] Thus, tubulin exhibits significant formation of isoaspartyl sites under conditions of physiological pH and temperature.

In vitro aging of purified tubulin at neutral pH has been reported to result in aggregation via the formation of disulfide bonds, a lesser degree of cross-linking via the formation of lysinoalanine, and to degradation via peptide bond cleavage (Correia et al., 1993; Prakash & Timasheff, 1982). The possible degradation of tubulin by spontaneous cleavage or by contaminating proteases was of particular concern to us

[2] Mammalian cells in general (Nuccitelli & Heiple, 1981), and rat PC12 cells in specific (Maduh et al., 1990), are known to have a cytosolic pH of 7.2 ± 0.1 under most conditions. We used a pH of 7.4 in this study so that our results could be compared directly with several previous studies (done at pH 7.4, 37 °C) on the rates of isoaspartate formation in synthetic peptides and purified proteins (Johnson & Aswad, 1995).

[3] If the isoaspartate accumulation were due only to first-order degradation of a single Asn or Asp site, the accumulation of methyl-accepting sites should not appear to be linear beyond 0.3 mol of CH_3/mol of tubulin; rather, the stoichiometry should level off, approaching an asymptote at 1.0 mol/mol. The roughly linear kinetics up to 0.8 mol/mol seen in Figure 1 suggest that there are multiple sites of isoaspartate formation. Indeed, "linear" kinetics of methyl-accepting site accumulation have been seen up to a high stoichiometry with in vitro aging of recombinant human growth hormone (Johnson et al., 1989b), mammalian calmodulin (Johnson et al., 1987a), and recombinant human tissue plasminogen activator (Paranandi et al., 1994). In all three cases, isoaspartate was found to accumulate at multiple sites, with no single site contributing more than 40−50% of the total.

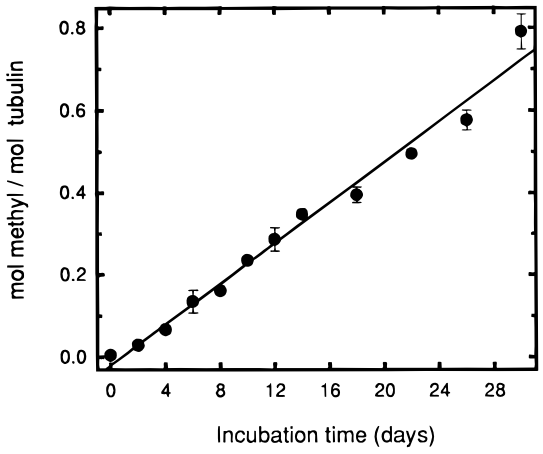

FIGURE 3: Effect of in vitro aging on the methyl-accepting capacity of tubulin. Purified rat brain tubulin was dialyzed against a pH 7.4 buffer containing 50 mM K-HEPES, 1 mM EGTA, 5% glycerol, and 0.02% sodium azide. It was then adjusted to a concentration of 1.0 mg/mL protein by dilution with the same buffer and incubated at 37 °C. At the times indicated on the abcissa, samples were removed and assayed at pH 6.8 for PIMT-dependent methyl-accepting capacity by the methanol diffusion method as described by Paranandi et al. (1994). Methylation reactions were carried out for 30 min at 30 °C and contained 0.8 μM PIMT and 2.0 μM tubulin. The methanol diffusion assay used here assures that only carboxyl methylation is measured (Najbauer et al., 1991). Thus, even if the tubulin was contaminated with lysine- or arginine-specific methyltransferases, methylation of these amino acids would not have contributed to the data in this figure.

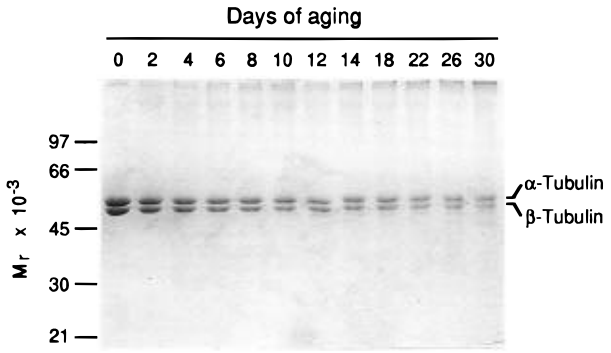

FIGURE 4: Effect of in vitro aging on the molecular mass of purified tubulin monitored by alkaline SDS−PAGE. Samples of aged tubulin (see legend to Figure 3) were subjected to SDS−PAGE (after heating in the presence of β-mercaptoethanol) according to Laemmli and Favre (1973). Samples containing 2.0 μg of tubulin were loaded in each lane. In parallel with the progressive loss of Coomassie staining of α- and β-tubulin at 50 kDa, there is increased staining in the upper third of each lane indicative of non-disulfide cross-linking.

given the extensive period of incubation used in our aging protocol. Degradation of tubulin would undermine the significance of isoaspartate formation in tubulin since flexible peptides tend to form isoaspartate much more readily than structured proteins. To test for degradation, samples of tubulin taken during the incubation were heated in 2% SDS containing 3% β-mercaptoethanol and subjected to SDS−PAGE in the alkaline Tris−glycine system of Laemmli and Favre (1973). As shown in Figure 4, there is little evidence for tubulin degradation, but there is a noticeable decrease with time in the intensity of protein staining at the positions of the α- and β-tubulin subunits. In parallel with this decrease, there is increased staining of diffuse bands in the molecular mass region of 100 kDa and above. The generation of nonreducible aggregates of tubulin during in vitro aging has been reported recently by Correia et al. (1993), and was attributed to the formation of lysinoalanine cross-links.

Although the widely used Tris−glycine SDS−PAGE system gives a high-resolution separation of proteins according to molecular weight, the alkaline pH of this system precludes analysis of many PIMT-methylated proteins because the methyl esters formed at isoaspartyl sites are generally unstable at neutral and alkaline pH. To determine the molecular weights of the methyl-accepting forms of tubulin, we analyzed methylated samples of aged tubulin by SDS−PAGE at pH 2.4. A fluorogram of our first analysis in this gel system is shown in Figure 5A. Samples of methylated tubulin were prepared for electrophoresis by heating them at 50 °C for 10 min in a pH 2.4 loading buffer that contained 2% SDS and 50 mM DTT. Nearly all of the methyl label was associated with highly aggregated material running at the top of the gel. Since it is well established that tubulin readily forms intermolecular disulfide cross-links during in vitro aging under conditions similar to ours (Correia et al., 1987, 1993, Prakash & Timasheff, 1982), we reasoned that the low pH of the standard loading buffer had not allowed effective reduction of disulfide cross-linked tubulin. This experiment was then repeated after changing the pH of the loading buffer to 6.2 (Figure 5B). With this modification, the majority of the methyl label was associated with 50 kDa tubulin bands, although significant methylation was still associated with high molecular weight material. As judged most easily by the 6−12 day time points, the relative contribution and diffuse banding pattern of the high molecular weight methyl acceptors were similar to the pattern of protein staining seen in Figure 4, suggesting that this methylation was associated with nonreducible aggregates of tubulin.

The Coomassie stain pattern of the gel corresponding to Figure 5A (not shown) indicated that disulfide cross-linking of tubulin was virtually complete during the first 2 days of incubation; thus, nearly all of the stain had shifted from the 50 kDa position to the top of the gel, coincident with the position of the methyl-accepting bands seen in Figure 5A. This raised a question as to whether isoaspartate forms only in aggregated tubulin, or whether it is an independent event. To address this question, we investigated the effect of 5 mM DTT on tubulin aging. Figure 5C shows the methylation pattern of tubulin after aging for 6 days in the presence (+) or absence (−) of DTT. Both samples were then prepared for electrophoresis exactly as described for panel A; that is, they were heated in loading buffer at pH 2.4, conditions that do not reduce disulfide bonds. The methylation pattern of the +DTT samples is virtually identical to that of the 6 day aged tubulin in panel 5B. This indicates that isoaspartate formation occurs independently of disulfide cross-linking.

Evidence for Generation and Repair of Isoaspartyl Sites in Tubulin in Vivo. If tubulin undergoes PIMT-dependent repair of isoaspartyl sites in vivo, then inhibition of PIMT in cultured cells should lead to an increase in the methyl-accepting capacity of the tubulin. To test this prediction, PC12 cells were incubated for 3 days in the presence or absence of 10 μM AdOx. Tubulin-enriched extracts were prepared by taxol precipitation of the 48000*g* supernatant fraction according to method A under Experimental Procedures. The post-taxol, tubulin-rich pellets were resuspended

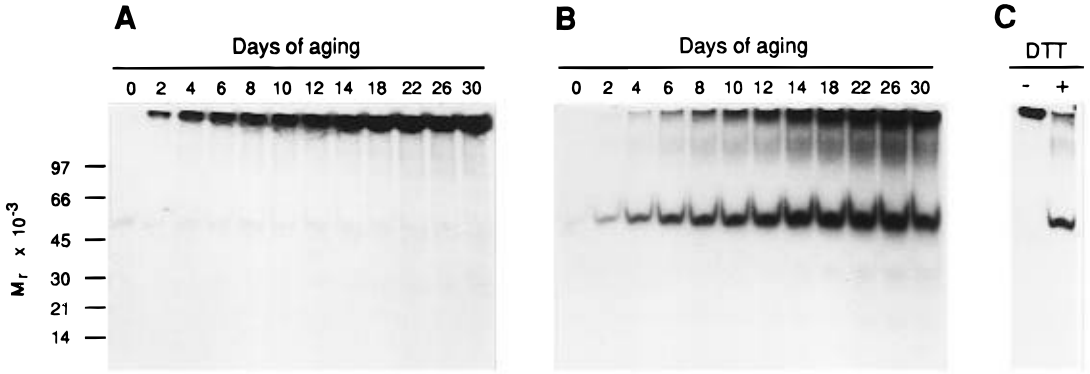

FIGURE 5: Molecular masses of methyl-accepting forms of tubulin generated by in vitro aging. Samples of tubulin (aged according to the legend for Figure 3) were methylated at pH 6.2 for 30 min, as described under Experimental Procedures, prior to electrophoresis in the pH 2.4 SDS−PAGE system. In panel A (unreduced samples) the methylation reactions were stopped by combining 6 μL of aged tubulin sample with 2 μL of 4× concentrated acidic SDS−PAGE sample buffer (200 mM sodium phosphate, pH 1.8, 8% SDS, 0.12% pyronin-Y, 40% glycerol, and 40 mg/mL DTT). Stopped samples were heated at 50 °C for 10 min prior to electrophoresis. Because of the low pH, reduction of disulfide bonds did not take place. In panel B (reduced samples), the methylation reactions were stopped by combining the tubulin sample with a modified 4× sample buffer from which the pH 1.8 sodium phosphate buffer had been omitted, allowing the stopped samples to remain at pH 6.2 during the 10 min heating at 50 °C. Subsequently, the pH 1.8 buffer was added, and the samples were loaded for electrophoresis. In panel C, tubulin was aged for 6 days as described in the legend to Figure 3, except that 5 mM DTT was present in the aging buffer of the +DTT sample to minimize disulfide bond formation. After aging, these samples were methylated and processed for electrophoresis in the same manner as the samples in panel A.

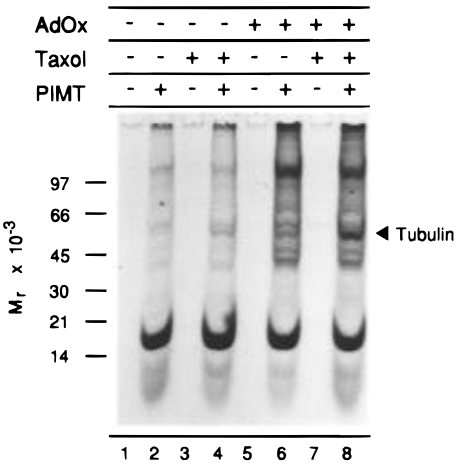

FIGURE 6: Accumulation of isoaspartyl sites in tubulin of PC12 cells during methyltransferase inhibition; analysis of low-speed extracts by acidic SDS−PAGE. Rat PC12 cells were cultured in the presence (+) or absence (−) of 10 μM AdOx for 72 h. The taxol-promoted microtubule polymerization procedure was carried out on low-speed extracts (method A of Experimental Procedures) with (+) or without (−) the inclusion of taxol. Samples were methylated in the presence (+) or absence (−) of purified PIMT and subjected to acidic SDS−PAGE (1.2 μg protein per lane). A fluorogram (3 month exposure) of the gel is shown. The position of an ^{3}H-methylated sample of aged rat brain tubulin run on the same gel is indicated on the right. A comparison of lanes 6 and 8 shows that the amount of ^{3}H-methylated protein comigrating with tubulin is much greater when taxol is present during the sample preparation. A comparison of lanes 4 and 8 indicates that the amount of methylation of tubulin (and of numerous other proteins with masses ≥40 kDa) is much greater in extracts from AdOx-treated cells than from control cells.

in pH 6.2 buffer and methylated by PIMT using *S*-adenosyl-L-[*methyl*-^{3}H]methionine to label the isoaspartyl sites. Methylation was carried out with high PIMT and AdoMet levels in order to maximize the probability that all available isoaspartyl sites were completely methylated. Figure 6 shows the fluorogram obtained after subjecting these samples to SDS−PAGE at pH 2.4. A comparison of lanes 4 and 8 shows that a number of proteins with molecular masses ≥40 kDa exhibit significantly increased methyl incorporation in response to AdOx treatment. Most prominent among these are proteins with apparent masses of 110 and 50 kDa. The 50 kDa protein was assumed to be tubulin since it comigrated with [^{3}H]methyl-labeled aged rat brain tubulin and because its level is selectively diminished when taxol is omitted (lane 6 vs lane 8). The AdOx effect suggests that tubulin constantly forms isoaspartyl sites in vivo, but that these sites are normally maintained at low steady-state levels by PIMT-dependent repair. When PIMT is inhibited, the steady-state level of isoaspartate rises due to the slower rate of repair. These results demonstrate that tubulin is one of several in vivo substrates for PIMT.

The most extensive methylation seen in Figure 6 occurs in a protein with an apparent molecular mass of 18−20 kDa whose methylation capacity is unaffected by treatment of the cells with AdOx. This relatively high level of isoaspartate, combined with the lack of AdOx effect, suggests that this protein may reside in a compartment or complex that is inaccessible to PIMT. The presence of this protein was previously noted in an earlier study from our lab on the effects of AdOx on PC12 proteins (Johnson et al., 1993).

To assess the reproducibility of the AdOx effect seen in Figure 6, a second set of tubulin-enriched extracts was prepared (via method B under Experimental Procedures) from 180000*g* supernatant fractions of quadruplicate control cultures or from quadruplicate cultures treated for 24 h with 10 μM AdOx. Samples of each extract were ^{3}H-methylated and analyzed by SDS−PAGE at pH 2.4 (Figure 7A). The results are similar to those seen in Figure 6; AdOx treatment resulted in a significant and reproducible increase in the methylation capacity of a protein that comigrates with purified tubulin. The main difference between Figures 6 and 7 is the lower level of background methylation in the latter. This can be attributed to the use of a higher speed supernatant fraction.

If AdOx treatment of PC12 cells were to cause significant changes in the tubulin content of the cells, or if it affected the purity of the tubulin prepared by the taxol method, then the results shown in Figures 6 and 7 could be construed as an artifact. To assess this possibility, we used the alkaline

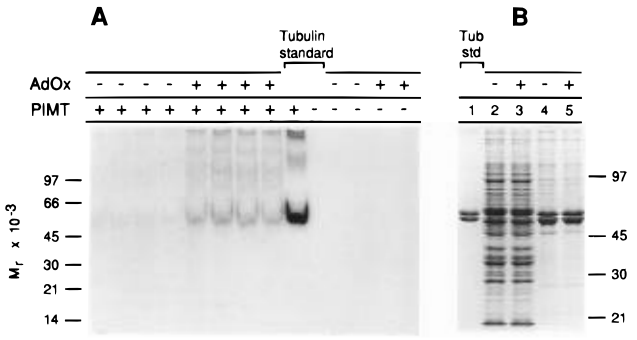

FIGURE 7: (A) Accumulation of methyl acceptor sites in tubulin of PC12 cells during methyltransferase inhibition; analysis of quadruplicate high-speed extracts. Rat PC12 cells were cultured in the presence (+) or absence (−) of 10 μM AdOx for 24 h. Quadruplicate cultures were used for each condition. The taxol-promoted microtubule polymerization procedure was carried out on high-speed extracts (method B of Experimental Procedures). Samples were methylated in the presence (+) or absence (−) of purified PIMT and subjected to acidic SDS−PAGE (4.7 μg of protein per lane). The fluorogram shown was obtained after an exposure of 7 days. Lanes containing ^{3}H-methylated samples of purified rat brain tubulin standard are indicated. The first eight lanes of panel A represent samples from eight independent cultures of PC12 cells. A comparison of the four (+) AdOx samples with the four control (−) samples confirms that AdOx treatment increases the amount of methyl-accepting tubulin. (B) Demonstration that AdOx treatment of PC12 cells does not alter the overall yield of tubulin in the procedures used in panel A. Protein staining of an alkaline SDS−PAGE is shown. Lane 1 contains 2.5 μg of the purified rat brain tubulin used throughout this study. Lanes 2 and 3 contain 20 μg of high-speed extract from control and AdOx-treated PC12 cells, respectively. Lanes 4 and 5 contain 10 μg each of the post-taxol tubulin-enriched fractions from high-speed extracts of control and AdOx-treated PC12 cells, respectively.

SDS−PAGE system (panel B of Figure 7) to analyze samples of the high-speed supernatant (lanes 2 and 3) and the final taxol-enriched tubulin preparation (lanes 4 and 5) that were used in panel A. The protein staining pattern shown in panel B indicates that AdOx treatment has no detectable effect on the yield or purity of the tubulin in the PC12 extracts.

Is Tubulin the Major Endogenous Substrate for PIMT in Rat Brain Cytosol? A number of years ago, this laboratory reported the presence of a major methyl acceptor in cytosol of calf brain with an apparent mass of 45−46 kDa as judged by CPC−PAGE (Aswad & Deight, 1983). Subsequently, Ohta et al. (1987) reported that tubulin was one of three major methyl acceptors present in the cytosol of rat brain. They found that tubulin ran with an apparent mass of 43 kDa during gel electrophoresis in the presence of the cationic detergent 16-BAC, and suggested that our previously reported 46 kDa methyl acceptor was tubulin.

To evaluate this suggestion, we used a 96000*g* cytosol fraction of rat brain to compare its PIMT-dependent methylation profile in the pH 2.4 SDS−PAGE system to the migration of purified tubulin. As shown by a comparison of lanes 3 and 4 in Figure 8A, the major methyl acceptor from rat brain cytosol has an apparent mass of 45 kDa and does not comigrate with a methylated tubulin standard (lane 4 vs lane 5). PIMT-dependent methylation of a weaker band comigrating with tubulin is evident in lanes 4 and 5, but its apparent mass is about 5 kDa higher than that of the "major" methyl acceptor.

A possible explanation for the discrepancy between our findings, and those of Ohta and colleagues, is provided in panel B of Figure 8, where the same set of samples used in

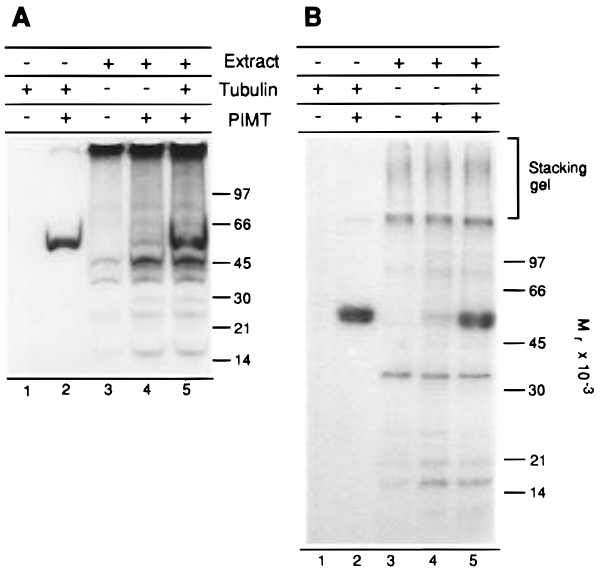

FIGURE 8: Substrates for PIMT in rat brain cytosol. Purified rat brain tubulin (unaged) and/or rat brain cytosol were ^{3}H-methylated at 30 °C for 40 min in the presence (+) or absence (−) of added PIMT (1.3 μM). Tubulin and brain cytosol were present (where indicated) in the methylation reactions at concentrations of 2.7 μM and 4.0 mg/mL, respectively. Proteins were then separated by SDS−PAGE under acidic (panel A) or alkaline (panel B) conditions. Alkaline SDS−PAGE was carried out at 4 °C for this analysis. Fluorograms of the dried gels are shown. In lane 4 of panel A, it is evident that the dominant PIMT-dependent methyl acceptor in rat brain cytosol runs at 45−46 kDa and does not comigrate with the tubulin standard. There is, however, a band of lesser intensity that does comigrate with tubulin. In lane 4 of panel B, there is barely detectable methylation of protein in the 45−45 kDa region, whereas methylation of a band comigrating with tubulin is clearly evident.

panel A was subjected to SDS−PAGE in the alkaline Tris−glycine buffer. In this electrophoresis system, the majority of protein methyl esters are destroyed by hydrolysis. This is expected because isoaspartyl sites tend to occur most frequently at succinimide-prone sequences in which the C-flanking amino acid is Gly, Ser, or His; thus, the remaining pattern of protein methylation will be heavily biased toward proteins whose methyl esters are atypically stable. Interestingly, under these conditions, tubulin appears as the most easily detectable PIMT-dependent methyl acceptor in the 40−60 kDa mass range (lane 4 in panel B). Methylation of a distinct 45−46 kDa protein is barely detectable. The significant methylation of purified tubulin observed in lanes 2 and 5 of panel 8B demonstrates that a portion of the isoaspartyl sites formed via in vitro aging are atypically stable. Although Ohta and colleagues used an acidic electrophoresis system to separate methylated proteins, it is possible that their methylated extracts or gels were inadvertently subjected to a combination of time, temperature, and pH that allowed significant hydrolysis of protein methyl esters prior to fluorography. This would have biased their methylation profile toward one that overemphasizes proteins containing atypically stable methylation sites.

DISCUSSION

The findings presented here suggest that tubulin is an important physiological substrate for PIMT. In untreated PC12 cells, a low but detectable level of isoaspartate is present in tubulin. When methyltransferase activity is inhibited, the level of isoaspartate rises significantly, con-

sistent with the idea that PIMT activity is necessary to keep isoaspartate from accumulating to high levels in tubulin. We previously demonstrated that methyltransferase inhibition causes a general increase in isoaspartate levels in PC12 cell proteins. The present work represents the first example of a specific protein that contributes to this effect. Thus, tubulin appears to be one of several proteins that constantly undergo formation and repair of atypical isoaspartyl linkages in vivo.

Not all proteins that form isoaspartate in vivo are necessarily substrates for PIMT. A case in point is the 18−20 kDa protein observed in Figure 6 and noted previously. This protein appears to have the highest levels of isoaspartate of any protein present in soluble extracts of untreated PC12 cells, but the level of isoaspartate does not change when PIMT activity is inhibited. A likely explanation is that this protein is sequestered in the cell in such a way that it is not accessible to PIMT. An inability of PIMT to gain access to this protein would explain both its lack of change in methylation capacity upon inhibitor treatment and why this protein is so enriched in isoaspartyl sites in the first place.

Tubulin plays a number of essential roles in cell function including maintenance and dynamic control of cell shape, movement of chromosomes during cell division, axonal transport, and bending of flagella and cilia. The formation of isoaspartate in proteins such as calmodulin and epidermal growth factor has been shown to result in a significant loss of function [reviewed by Teshima et al. (1995)]. Likewise, isoaspartate formation in polymerized tubulin may disrupt the function of the microtubule. Alternatively, isoaspartate formation within the 50 kDa subunits of tubulin might prevent the formation of a functional α/β dimer, or the incorporation of the dimer into a growing microtubule. In either case, the expenditure of a few AdoMet molecules to rescue a damaged subunit presents a clear savings in cell energy compared with degradation and replacement of an entire tubulin subunit. The importance of tubulin as a target for PIMT action is consistent with the known distribution of these two proteins in mammalian cells. The highest specific activities of PIMT are found in brain and testes (Diliberto & Axelrod, 1976). The high level in testes has been attributed to its high concentration in sperm tails (Bouchard et al., 1980), a region where microtubules constitute a significant portion of the ultrastructure. The abundance of microtubules in the brain (where it is found in axons as well as in neuronal and glial cell bodies) is well documented.

The formation of isoaspartate in tubulin within cultured PC12 cells is corroborated by the finding that purified tubulin generates isoaspartate at a significant rate in vitro at physiological pH and temperature. Studies on the in vitro formation of isoaspartate in purified proteins and peptides indicate that it forms most readily at Asn-Gly, Asn-Ser, Asn-His, and Asp-Gly sites when these sequences fall in flexible domains of the polypeptide (Johnson & Aswad, 1995; Lowenson & Clarke, 1995; Oliyai & Borchardt, 1994; Patel & Borchardt, 1990; Tyler-Cross & Schirch, 1991). We used the flexibility plot of Ragone et al. (1989) to determine if tubulin contains any isoaspartate-prone sequences in regions that are predicted to be flexible. As shown in Figure 9, α-tubulin has two Asp-Gly sequences (positions 33−34 and 245−246) that are in regions predicted to be highly flexible while β-tubulin has two Asn-Ser sequences (positions 337−338 and 370−371) that are predicted to be flexible. It is likely that one or more of these four sites contributes significantly to the generation of isoaspartate in aged tubulin.

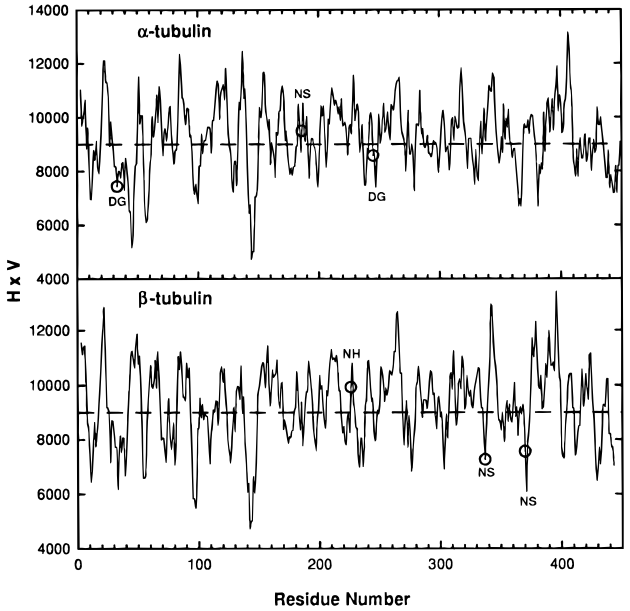

FIGURE 9: Flexibility plots of the α and β forms of rat tubulin. Flexibility is plotted according to Ragone et al. (1989) using a five-residue moving sum of *H* (hydrophobicity index) × *V* (side chain volume index). In this plot, lower values mean greater predicted flexibility. Most proteins, including tubulin, show an overall average *HV* of approximately 9000 (dashed line). The positions of amino acid sequence pairs found to be highly susceptible to succinimide formation in model peptides under mild conditions (NG, NS, NH, and DG) are indicated by labeled circles.

In addition to isoaspartate formation, tubulin undergoes two additional types of covalent modification during in vitro aging: formation of disulfide cross-links between subunits and another form of aggregation that apparently does not involve disulfide bonds. The occurrence of these two types of aggregation during in vitro aging of tubulin has been reported previously by Correia and colleagues (Correia et al., 1987, 1993), who suggested that the nonreversible aggregation occurs via formation of lysinoalanine cross-links. Lysinoalanine is known to form in proteins as a consequence of spontaneous β-elimination of amino acids such as serine, phosphoserine, or cystine, to form dehydroalanine (Bohak, 1964; Chang, 1991; Friedman, 1994; Jones et al., 1983). The β-carbon of dehydroalanine then reacts with the side ϵ-amino group of a lysine to form the lysinoalanine cross-link. Since the cross-linkage is made through a secondary amine bond, lysinoalanine remains intact during acid hydrolysis of the protein, greatly facilitating the detection of this type of cross-link. Correia and co-workers detected 0.13 mol of lysinoalanine formed per mole of tubulin after incubation of purified porcine tubulin at 35 °C, pH 10, for 3 days (Correia et al., 1993).

Since cyclic imides are highly susceptible to reaction with nucleophiles, it seems possible that a portion of the cross-linking observed during in vitro aging of tubulin at pH 7.4 results from formation of aspartyllysine cross-links. As demonstrated in Figure 5B, the rate of cyclic imide formation (deduced from the rate of generation of methylation sites) in tubulin closely parallels the rate of irreversible aggregation. The data in Figure 3 indicate that tubulin generates cyclic imides in vitro at a rate of at least 3.4 mol % per day.[4] Moreover, in model peptides, cyclic imides exhibit half-lives

of 2−4 h at pH 7.4, 37 °C (Geiger & Clarke, 1987; Johnson & Aswad, 1985; Murray & Clarke, 1986), providing sufficient time for the imide to react with a lysine side chain. Cross-links of this type have been observed in proteolytic digests of heat-treated proteins (Otterburn et al., 1977) and in human colostrum (Klostermeyer, 1984). We recently reported (Paranandi & Aswad, 1995) that in vitro aging of purified synapsin-1 from cow brain results in isoaspartate formation, disulfide cross-linking, and irreversible aggregation, with a kinetic profile similar to that observed with tubulin. Tubulin and synapsin would thus appear to be excellent candidates for investigating the possibility that cyclic imide formation may play a role in the spontaneous cross-linking of proteins under mild conditions. Disulfide bond formation in these proteins may enhance the rate of irreversible cross-linking by bringing subunits close together, thereby increasing the probability that the lysine side chain of one molecule will react with a dehydroalanine or succinimide in another molecule.

REFERENCES

Aswad, D. W. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., Ed.) pp 31−46, CRC Press, Boca Raton, FL.

Aswad, D. W., & Deight, E. A. (1983) *J. Neurochem. 41*, 1702−1709.

Aswad, D. W., & Johnson, B. A. (1987) *Trends Biochem. Sci. 12*, 155−158.

Bartel, R. L., & Borchardt, R. T. (1984) *Mol. Pharmacol. 25*, 418−424.

Bohak, Z. (1964) *J. Biol. Chem. 239*, 2878−2887.

Bornstein, P., & Balian, G. (1977) *Methods Enzymol. 47*, 132−145.

Bouchard, P., Gagnon, C., Phillips, D. M., & Bardin, C. W. (1980) *J. Cell Biol. 86*, 417−423.

Brennan, T. V., & Clarke, S. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., Ed.) pp 65−90, CRC Press, Boca Raton, FL.

Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B., & Clarke, S. (1994) *J. Biol. Chem. 269*, 24586−24595.

Chamberlain, J. P. (1979) *Anal. Biochem. 98*, 132−135.

Chang, J.-Y. (1991) *J. Biol. Chem. 266*, 10839−10843.

Correia, J. J., Welch, M. K., & Williams, R. C., Jr. (1987) *Arch. Biochem. Biophys. 255*, 244−253.

Correia, J. J., Lipscomb, L. D., & Lobert, S. (1993) *Arch. Biochem. Biophys. 300*, 105−114.

Diliberto, E. J., Jr., & Axelrod, J. (1976) *J. Neurochem. 26*, 1159−1165.

Fairbanks, G., & Avruch, J. (1972) *J. Supramol. Struct. 1*, 66−75.

Friedman, M. (1994) *J. Agric. Food Chem. 42*, 3−20.

Galletti, P., Ciardiello, A., Ingrosso, D., & Di Donato, A. (1988) *Biochemistry 27*, 1752−1757.

Geiger, T., & Clarke, S. (1987) *J. Biol. Chem. 262*, 785−794.

Henzel, W. J., Stults, J. T., Hsu, C.-A., & Aswad, D. W. (1989) *J. Biol. Chem. 264*, 15905−15911.

Hoffman, J. L. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 181−186, Elsevier North Holland, New York.

Johnson, B. A., & Aswad, D. W. (1985) *Biochemistry 24*, 2581−2586.

Johnson, B. A., & Aswad, D. W. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., Ed.) pp 91−114, CRC Press, Boca Raton, FL.

Johnson, B. A., Langmack, E. L., & Aswad, D. W. (1987a) *J. Biol. Chem. 262*, 12283−12287.

Johnson, B. A., Murray, E. D. Jr., Clarke, S., Glass, D. B., & Aswad, D. W. (1987b) *J. Biol. Chem. 262*, 5622−5629.

Johnson, B. A., Shirokawa, J. M., & Aswad, D. W. (1989a) *Arch. Biochem. Biophys. 268*, 276−286.

Johnson, B. A., Shirokawa, J. M., Hancock, W. S., Spellman, M. W., Basa, L. J., & Aswad, D. W. (1989b) *J. Biol. Chem. 264*, 14262−14271.

Johnson, B. A., Najbauer, J., & Aswad, D. W. (1993) *J. Biol. Chem. 268*, 6174−6181.

Jones, A. J., Helmerhorst, E., & Stokes, G. B. (1983) *Biochem. J. 211*, 499−502.

Klostermeyer, H. (1984) *Methods Enzymol. 107*, 258−261.

Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 575−599.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335−341.

Li, C., & Clarke, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9885−9889.

Lowenson, J. D., & Clarke, S. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., Ed.) pp 47−64, CRC Press, Boca Raton, FL.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.

Maduh, E. U., Borowitz, J. L., & Isom, G. E. (1990) *Toxicol. Appl. Pharmacol. 106*, 201−208.

McFadden, P. N., & Clarke, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2595−2599.

Murray, E. D. Jr., & Clarke, S. (1986) *J. Biol. Chem. 261*, 306−312.

Najbauer, J., & Aswad, D. W. (1990) *J. Biol. Chem. 265*, 12717−12721.

Najbauer, J., Johnson, B. A., & Aswad, D. W. (1991) *Anal. Biochem. 197*, 412−420.

Najbauer, J., Johnson, B. A., Young, A. L., & Aswad, D. W. (1993) *J. Biol. Chem. 268*, 10501−10509.

Nuccitelli, R., & Heiple, J. M. (1981) in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 567−586, Alan R. Liss, Inc., New York.

Ohta, K., Seo, N., Yoshida, T., Hiraga, K., & Tuboi, S. (1987) *Biochimie 69*, 1227−1234.

Oliyai, C., & Borchardt, R. T. (1994) *Pharm. Res. 11*, 751−758.

Otterburn, M., Healy, M., & Sinclair, W. (1977) *Adv. Exp. Biol. Med. 86B*, 239−262.

Paranandi, M. V., & Aswad, D. W. (1995) *Biochem. Biophys. Res. Commun. 212*, 442−448.

Paranandi, M. V., Guzzetta, A. W., Hancock, W. S., & Aswad, D. W. (1994) *J. Biol. Chem. 269*, 243−253.

Patel, K., & Borchardt, R. T. (1990) *Pharm. Res. 7*, 787−793.

Potter, S. M., Johnson, B. A., Henschen, A., & Aswad, D. W. (1992) *Biochemistry 31*, 6339−6347.

Potter, S. M., Henzel, W. J., & Aswad, D. W. (1993) *Protein Sci. 2*, 1648−1663.

Prakash, V., & Timasheff, S. (1982) *J. Mol. Biol. 160*, 499−515.

Ragone, R., Facchiano, A., Facchiano, A. M., & Colonna, G. (1989) *Protein Eng. 2*, 497−504.

Sharma, S., Hammen, P. K., Anderson, J. W., Leung, A., Georges, F., Hengstenberg, W., Klevit, R., & Waygood, E. B. (1993) *J. Biol. Chem. 268*, 17695−17704.

Teshima, G., Hancock, W. S., & Canova-Davis, E. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., Ed.) pp 167−192, CRC Press, Boca Raton, FL.

Tyler-Cross, R., & Schirch, V. (1991) *J. Biol. Chem. 266*, 22549−22556.

Vallee, R. B. (1986) *Methods Enzymol. 134*, 89−104.

Williams, R. C., Jr., & Lee, J. C. (1982) *Methods Enzymol. 85*, 376−385.

Wright, H. T. (1995) in *Deamidation and Isoaspartate Formation in Peptides and Proteins* (Aswad, D. W., Ed.) pp 229−252, CRC Press, Boca Raton, FL.

[4] This is calculated from the rate of isoaspartate accumulation seen in Figure 3, 2.4%/day, divided by 0.7, the mole fraction of isoaspartate formed upon succinimide hydrolysis (Figure 2).

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