High Mass Methyl-accepting Protein (HMAP), a Highly Effective Endogenous Substrate for Protein 1-Isoaspartyl Methyltransferase in Mammalian Brain*

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Communication

A previously unidentified endogenous substrate for protein 1-isoadsartyl methyltransferase in mammalian brain has been characterized and partially purified. This high mass methyl-accepting protein (HMAP) is concentrated in rat brain cytosol and is not detectable in rat liver, heart, lung, kidney, or skeletal muscle. HMAP is acidic and heterogeneous in size, with an average mass, as judged by size-exclusion high performance liquid chromatography, greater than 700 kDa. After partial purification from cow brain by anion-exchange chromatography, ammonium sulfate fractionation, and gel filtration, HMAP could accept 12.1 nmol of methyl groups per mg of protein, suggesting that it contains a level of isoaspartate at least 50 times greater than that of the average protein in brain cytosol. Partially purified HMAP is degraded by trypsin, verifying that it is composed, at least in part, of protein. Additional studies on this unusual macromolecule may shed important new light on mechanisms of isoaspartate formation in cells and the molecular pathology of brain aging.

Formation of atypical β-linked isopeptide bonds at Asp-X and Asn-X sequences is emerging as a major source of spontaneous protein damage under physiological conditions (1–4). Formation of isoaspartate occurs when the α-nitrogen of the C-flanking amino acid (X) attacks the side-chain carbonyl of an Asp or Asn residue, resulting in the formation of a succinimide intermediate. The succinimide subsequently hydrolyzes to produce a mixture typically composed of 15–30% aspartate in a normal α-linkage and 60–85% aspartate in an abnormal β-linkage (isoAsp). Asp-X and Asn-X sequences both make significant contributions to isoAsp formation during in vitro aging of purified proteins. This reaction can account for the observation that internal Asn residues tend to deamidate more rapidly under mild conditions than internal Gln residues (5).

Protein-1-isoadsartyl O-methyltransferase (PIMT; EC 2.1.1.77), an enzyme found in bacteria, plants, and animals (6, 7), catalyzes the S-adenosyl-L-methionine (AdoMet)-dependent methylation of the free α-carboxyl of 1-isoadsartyl residues. At physiological pH, this methylation initiates conversion of isoAsp linkages back to normal Asp linkages in both peptides and proteins, suggesting that PIMT may serve as a repair enzyme in vivo (8–12). PIMT-dependent methylation of isoAsp sites with [methyl-3H]AdoMet also provides a convenient assay for quantitating levels of isoAsp in peptides and proteins (13). The widespread phylogenetic distribution of this enzyme suggests that formation of isoAsp is a common mechanism of protein damage in vivo.

Brain is one of the richest sources of PIMT in mammalian tissues (14, 15). In a recent study on formation of isoAsp in rat brain tubulin (16), we noted the presence of a high mass methyl-acceptor (>200 kDa) that was labeled extensively when brain cytosol was incubated in the presence of [methyl-3H]AdoMet. Initial experiments suggested that the methylation of this molecule was not dependent on the addition of purified PIMT, implying that it might be a substrate for a different methyltransferase. We observed, however, that methylation of this molecule was partially reversed by exposure to mildly alkaline conditions, a characteristic of many isoAsp methyl esters. In the present work, we provide direct evidence that this methyl acceptor, now termed HMAP (high mass methyl-accepting protein) is a specific and highly effective substrate for PIMT, and, moreover, that it is selectively enriched in brain cytosol. A partial purification and characterization of HMAP from cow brain is also presented.

EXPERIMENTAL PROCEDURES

Materials—S-Adenosyl-L-methionine, 15 Ci/mmole, was obtained from DuPont NEN and adjusted to lower specific activity by dilution with purified unlabeled AdoMet (Sigma). Recombinant rat PIMT (type I isoform) was isolated and purified to homogeneity as described previously (17). The specific activity of PIMT was 9000 units/mg, where a unit is defined as 1 pmol of methyl transferred per min to bovine γ-globulins (5 mg/ml) at pH 6.2, 30 °C. The normal and isoAsp-5 forms of β-sleep-inducing peptide (DSIP; Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) were obtained from Bachem Bioscience, Inc. Trypsin (type XIII) was obtained from Sigma. Leupeptin was obtained from Boehringer Mannheim.

Fractionation of Rat Brain—Immediately after decapitation, the brains of male Sprague-Dawley rats (7–8 weeks of age) were excised and homogenized at 4 °C in 4 volumes of fractionation buffer using six strokes of a Teflon-glass homogenizer rotating at approximately 300 rpm. Fractionation buffer consisted of 10% (w/v) sucrose dissolved in PEM buffer (5 mM sodium phosphate, pH 7.0, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.9 mg/ml leupeptin). Subsequent procedures were all carried out at 2–4 °C. The homogenate was filtered through cheesecloth, then centrifuged at 800 × g for 20 min to produce a crude nuclear fraction (P1). The first supernatant (S1) was centrifuged at 9,000 × g for 20 min to produce an intermediate pellet (P2). This second supernatant (S2) was then centrifuged at 100,000 × g for 60 min to produce a high-speed pellet (P3) and cytosol (S3). Prior to methylation analysis, the P1, P2, P3, and partial cytosol supernatant fractions were resuspended in fractionation buffer and boiled for 5 min.

Declaration of Interest—The authors declare no conflict of interest.

1 The abbreviations used are: PIMT, protein 1-isoadsartyl methyltransferase; AdoMet, S-adenosyl-L-methionine; BisTris, bis(2-hydroxyethyl)iminomorpholinoethane-N,N,N′-trisulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; DSIP, β-sleep-inducing peptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu); DTG, di(2-thioctyl)glycerol; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; HMAP, high mass methyl-acceptor protein; HPLC, high performance liquid chromatography; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid).

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and P3 fractions were washed twice by homogenization in fractionation buffer followed by centrifugation at the appropriate speed. Each of the washings was done in a volume of buffer twice that used for the original tissue homogenization. The twice-washed pellets were rapidly resuspended by homogenization in sucrose-free PEMPL buffer, to effect a hypotonic lysis with the intent of releasing HMAP trapped in vesiculated compartments. After 30 min in the hypotonic buffer, sucrose was added to a final concentration of 10% (w/v) prior to storage at −70 °C. The protein concentrations of the P1, P2, P3, and SI fractions were 7.8, 8.3, 5.4, and 4.4 mg/ml, respectively. Protein concentrations were determined with the method of Lowry et al. (18) after precipitation in 7% (w/v) trichloroacetic acid.

Methylation—Methylation reactions destined for analysis by SDS-PAGE were carried out in 6 μl of 75 mM potassium MES, pH 6.2, containing 0.25–3.0 mg/ml tissue-extract protein, and 50 μM [3H]AdoMet (20,000 dpm/pmol). When added, purified PIMT was present at 1.8 μM. Methylation was initiated by addition of the labeled AdoMet and carried out for 10 min at 30 °C. Reactions were stopped by adding 2 μl of stop solution (8% (w/v) SDS and 20% (w/v) 2-mercaptoethanol) followed by heating at 50 °C for 10 min. The stopped samples were prepared for acidic SDS-PAGE by adding 2 μl of 0.5 M sodium phosphate, pH 2.4, containing 50% (w/v) glycerol and 0.05% (w/v) pyronin Y.

Pre-column methylation of rat brain cytosol (S3) for analytical size-exclusion high performance liquid chromatography (HPLC) was carried out as above in a reaction volume of 30 μl. The reaction was terminated by combining it with 50 μl of unmethylated rat brain cytosol (13 mg/ml in PBSM buffer) to act as carrier, followed immediately by rapid gel filtration on a 1-ml Sephadex G25 (superfine) “spin-column” (19) equilibrated in 10 mM sodium phosphate, pH 6.2, 150 mM NaCl, and 1.5 mM 2-mercaptoethanol (PBSM buffer).

Post-column methylation of fractions from the size-exclusion HPLC used for analysis of trypsin sensitivity of HMAP was carried out at pH 6.8 for 30 min and processed by the diffusion assay as described previously (20).

SDS-PAGE and Fluorography—SDS-PAGE was carried out in 0.75-mm-thick slab gels in a continuous pH 2.4 buffer system (21). Gels of 10% or 5% acrylamide were used as indicated. Electrophoresis was carried out at room temperature in a Bio-Rad Mini-PROTEAN II apparatus at 50 V for 2.5 h. Molecular weight standards for SDS-PAGE were purchased from Bio-Rad. After electrophoresis, proteins were stained with Coomassie Blue R-250, impregnated with salicylate, dried, and then subjected to fluorography (40–50 h of exposure) on sensitized film as described previously (16).

Analytical Size-exclusion HPLC—To estimate the size of native HMAP, a 50-μl sample of methylated rat brain cytosol (400 μg of protein) was chromatographed on a 7.8 × 300 mm Bio-Sil TSK 125 (Bio-Rad) size-exclusion HPLC column. The column was equilibrated and run in PBSM buffer at a flow rate of 1.0 ml/min. Effluent was monitored at 278 nm while collecting fractions of 0.25 ml. The H content of each fraction was determined by scintillation counting. The column was calibrated with a set of gel filtration standards from Bio-Rad (catalog number 151-1901) consisting of thyroglobulin (670 kDa), immunoglobulin G (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The elution times of the standards were determined in a separate run under conditions identical to those used for analysis of the brain extracts. The elution pattern of the standard mixture, monitored at 278 nm, was virtually identical to the pattern provided by the manufacturer, thus allowing assignment of elution positions for each of the standards.

Purification of HMAP—Except where indicated otherwise, all procedures were carried out at 0–4 °C. Sixty grams of frozen, dehydrinellated, cow cerebral cortex were homogenized in 4 volumes of 20 mM BisTris-Cl, pH 7.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, and 0.5 μg/ml leupeptin for 1 min in an Ultra- turrax model STD Tissuemizer (Tekmar Co.). After centrifugation at 9,000 × g for 20 min, the supernatant was removed and recentrifuged at 100,000 × g for 1 h. This supernatant was adjusted to 0.25 M NaCl by addition of 1 M NaCl, then loaded onto a 2.5 × 20 cm column of DEAE-cellulose (Whatman DE23) previously equilibrated with 20 mM BisTris-Cl, pH 7.0, 0.25 M NaCl, 1 mM DTT. After washing with 1 column volume of this buffer, the column was developed with a 1-liter linear gradient of 0.25 to 1.0 M NaCl made up in the same buffer. HMAP (eluting as a single broad peak centered at about 0.5 M NaCl) was localized to column fractions by submitting a H-methylated portion of each fraction to acidic SDS-PAGE followed by fluorography. Fractions containing HMAP were pooled and brought to 30% saturation with solid ammonium sulfate. After removing precipitated proteins by centrifugation, the supernatant was adjusted to 95% saturation with additional ammonium sulfate, and the precipitate was collected by centrifugation. The final pellet was dissolved in 10 ml of sodium phosphate, pH 6.6, 0.25 M NaCl, 1.5 mM 2-mercaptoethanol, and loaded onto a 1.5 × 56 cm column of Sephacryl HR400 (Pharmacia Biotech Inc.) equilibrated with the same buffer and run at a flow rate of 1.0 ml/min. HMAP, detected as described above, eluted as a major peak between the positions of a 670-kDa marker and the void volume. Pooled fractions were concentrated to 4.0 ml (2.3 mg/ml protein) over an Amicon YM-10 membrane.

Tryptsin Treatment—HMAP, purified as above, was reduced and alkylated with iodoacetate as described previously (20). Approximately 6.9 mg of the reduced and alkylated HMAP was rechromatographed on the Sephacryl HR400 column, this time in the presence of 2.5 mM urea. Rechromatography indicated that reduction and alkylation had not significantly altered the molecular weight profile of HMAP. Ultrafiltration (Amicon YM-10 membrane) was used to simultaneously concentrate and exchange the rechromatographed HMAP into trypsin digestion buffer (0.1 M sodium acetate, 10 mM Tris acetate, 1 mM CaCl2, pH 8.0). Tryptsin digestion was carried out for 30 min at 37 °C in a 100-μl reaction containing 1.0 mg/ml HMAP and 0.05 mg/ml trypsin. After stopping the reaction with 2 μl of glacial acetic acid, two-thirds of the digest was applied to a 7.5 × 300 mm Bio-Sil TSK 125 (Bio-Rad) size-exclusion HPLC column run at 1.0 ml/min in 10 mM sodium acetate, pH 4.8, containing 150 mM NaCl. Effluent was monitored at 278 nm while collecting fractions of 0.5 ml. A sample of undigested HMAP, subjected to a mock trypsin incubation, was run under the same conditions to serve as a control.

Tissue Distribution of HMAP—The brain, heart, liver, lungs, kidneys, and leg muscle were excised from an 8-week-old male Sprague-Dawley rat. The leg muscle was homogenized for 30 s in 1 volume of cold 10% sucrose in PEMPL buffer using an UltraTurrax model STD Tissuemizer (Tekmar Co.). All the other tissues were homogenized in the same buffer using a Dounce homogenizer. The homogenates were centrifuged at 9,000 × g for 20 min. The resulting supernatants were then centrifuged at 100,000 × g for 60 min to produce the final cytosol. Samples of cytosol were then methylated in the presence or absence of purified PIMT as described above and analyzed by acidic SDS-PAGE in 10% acrylamide.

RESULTS

HMAP Is a Substrate for PIMT—As noted previously (16), incubation of rat brain cytosol with [methyl-3H]AdoMet, followed by SDS-PAGE in 10% acrylamide, results in a methylation pattern dominated heavily by a high mass macromolecule that barely enters the gel (Fig. 1A, lane 1). To determine if this methylation is dependent on the presence of endogenous PIMT, a parallel incubation was carried out in the presence of 0.4 mM isoAsp-DSIP, a commercially available isoAsp-containing nona-
peptide that serves as an effective substrate for both the rat recombinant \( K_m = 16 \mu M \) and the cow brain \( K_m = 4 \mu M \) enzymes (17, 22). The presence of this alternative substrate blocked methylation of HMAP (lane 2), while the presence of 0.4 mM DSIP (a homologous peptide containing a normal aspartate in place of the isoAsp) had no effect on endogenous methylation (lane 3). When purified PIMT was added to brain cytosol at a final concentration of 1.8 \( \mu M \), methylation of HMAP was stimulated to a level above that observed for endogenous methylation (lane 4 versus lane 1). These results indicate that HMAP is a highly effective and specific substrate for PIMT in rat brain extracts.

In an attempt to obtain a better estimate of the mass of HMAP, a sample similar to that used in lane 1 of Fig. 1A was subjected to SDS-PAGE in 5% acrylamide. As shown in Fig. 1B, HMAP migrated further into this gel, producing a diffuse region of methylation whose average apparent mass was well in excess of 200 kDa, indicating that HMAP is both large and heterogeneous.

Characterization and Partial Purification of HMAP—The subcellular distribution of HMAP in rat brain is shown in Table I. The highest specific activity was found in the S3 (100,000 \( \times g \) supernatant) fraction, accounting for 61% of total HMAP recovered from all fractions. The lysed P1 (900 \( \times g \) crude nuclear) fraction contained 32% of total HMAP. Much of this may represent soluble HMAP initially trapped in unbroken cells and large vesiculated cell fragments during homogenization.

To determine the apparent mass of undenatured HMAP, we subjected rat brain cytosol, methylated with or without addition of purified PIMT, to size-exclusion HPLC. As shown in Fig. 2A, methyl-accepting activity was dominated by a major peak eluting between 670 kDa and the column void volume. The preferential methylation of HMAP by endogenous PIMT shown in Fig. 1 prompted us to consider the possibility that HMAP might be tightly associated with PIMT in brain cytosol. To test this, we subjected unmethylated brain cytosol to size-exclusion HPLC and then assayed fractions for PIMT activity. All of the enzyme activity eluted between 9.7 and 10.8 min, consistent with the native molecular mass (25 kDa) of rat PIMT (data not shown); thus, any association of PIMT with HMAP in cytosol is insufficiently tight to persist during size-exclusion HPLC.

In preparation for undertaking a partial purification of HMAP, we determined that HMAP is present in cow brain at a level similar to that found in rat brain (data not shown). Because of its greater tissue mass, we chose to purify HMAP from cow brain. In preliminary experiments, we had found that rat HMAP bound to DEAE-cellulose, but not to CM-cellulose, at neutral pH and low ionic strength. Upon application of cow brain cytosol to DEAE-cellulose, we used a linear salt gradient to elute bound proteins from the column. HMAP eluted as a single broad peak centered at approximately 0.5 M NaCl, suggesting that it is a highly acidic protein (data not shown). By combining anion-exchange chromatography, ammonium sulfate fractionation, and gel filtration (see "Experimental Procedures"), we were able to purify cow brain HMAP approximately 50-fold (relative to cytosol) to a specific methyl-accepting activity of 12.1 nmol of CH\(_3\) incorporated per mg of protein.

Based on the total \([\text{H}]\)HMAP content of each fraction, measured in the presence of added PIMT.

### Table I

| Subcellular fraction | HMAP specific activity\( ^a \) pmol CH\(_3\)/mg protein | % of HMAP recovered from all fractions\( ^b \) | Endogenous +PIMT | lane 1 | lane 2 | lane 3 |
|----------------------|----------------------------------------------------------|---------------------------------------------|-----------------|--------|--------|--------|
| P1 (800 \( \times g \) pellet) | 0.9 | 16.7 ± 1.3 | 32.1 |
| P2 (9,000 \( \times g \) pellet) | 0.5 | 8.8 ± 0.9 | 5.0 |
| P3 (100,000 \( \times g \) pellet) | 0.0 | 12.5 ± 0.9 | 2.2 |
| S3 (cytosol) | 12.5 ± 0.4 | 58.4 ± 1.3 | 60.7 |

\( ^a \) HMAP activity was determined after SDS-PAGE as picomoles of \([\text{H}]\)CH\(_3\) recovered from each gel slice per mg of protein loaded per lane. Values listed are means ± S.E. of triplicate determinations.

\( ^b \) Based on the total \([\text{H}]\)HMAP content of each fraction, measured in the presence of added PIMT.

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**FIG. 2.** Size-exclusion HPLC of HMAP. A shows the profile of methyl-acceptors obtained when rat brain cytosol was methylated in the presence of added PIMT (upper panel) or in the presence of added isoAsp-DSIP (lower panel) and then chromatographed on a Bio-Sil SEC 250 size-exclusion column. Cytosol was methylated prior to loading onto the column. The positions of molecular weight markers are indicated at the top. B shows the effect of trypsin treatment on the size-exclusion behavior of HMAP partially purified from cow brain. Samples of HMAP, preincubated with (+) or without (−) trypsin, were loaded directly onto a Bio-Sil 125 column without prior methylation. Fractions were collected and assayed for methyl-accepting capacity in the presence of added PIMT by the methanol diffusion assay.
Endogenous Substrate for Brain Isoaspartyl Methyltransferase

**HMAP Is Highly Enriched in Brain**—To investigate the tissue distribution of HMAP, cytosol was prepared from freshly excised rat brain, heart, lung, liver, kidney, and skeletal muscle, methylated in the presence or absence of added PIMT or isoAsp-DSIP, and then analyzed by SDS-PAGE, followed by fluorography. As shown in Fig. 3, only brain contains significant levels of HMAP. Heart and lung tissues exhibit weak methylation of a large protein, but this substrate migrates with an apparent mass somewhat lower than that of HMAP. This result indicates that either HMAP is derived from a protein (or proteins) highly enriched in brain, or, alternatively, that HMAP is derived from a more widely distributed protein that is uniquely susceptible to isoaspartate formation in brain.

**DISCUSSION**

HMAP is extensively methylated in vitro by endogenous PIMT in brain cytosol and is the only such substrate detected under the conditions used in Fig. 1. This preferential methylation suggests that HMAP may have a high affinity for PIMT as well as an abundance of methyl-accepting sites. The specific methyl-accepting capacity of partially purified HMAP from cow brain was estimated to be 12.1 nmol of CH₃ per mg of protein, approximately 50 times the average methyl-accepting capacity of unfractionated brain cytosol. If one corrects for the observation that HMAP contributes about 70% of total methyl-accepting capacity seen in rat brain cytosol (Fig. 2A), then the methyl-accepting capacity of HMAP in cow brain is at least 150 times that of the average cytosolic protein (excluding HMAP itself). If HMAP were considered as an aggregate of 60-kDa subunits (a typical mass for a cytosolic protein), its methyl-accepting capacity would correspond to a stoichiometry of 0.70 mol of isoAsp per mol of 60-kDa subunit.

Mammalian PIMT has been shown to methylate both L-isoaaspartyl residues (10–12, 20) and D-aspartyl residues (23) in peptides and proteins; however, methylation of D-aspartyl residues in peptides seems to occur with a catalytic efficiency ($k_{cat}/K_m$) that is 2–3 orders of magnitude lower than L-isoaaspartyl residues (23). Under the conditions of our methylation assays, we would not expect significant methylation of D-aspartyl residues. Thus, it is reasonable to assume that the high methyl-accepting capacity of HMAP represents a high content of L-isoaaspartyl residues. Although they may not contribute significantly to methylation capacity under our conditions, the presence of D-aspartyl residues in HMAP would not be surprising since racemization of Asp occurs via formation of the same succinimide intermediate that generates peptide bond isomerization at Asp-X sequences (24). The high methyl-accepting capacity of HMAP, together with its large size and heterogeneity, suggest that it may consist of aggregates of damaged proteins that have accumulated high levels of L-isoaaspartyl (and possibly D-aspartyl) residues. Further purification and partial sequencing of HMAP peptides should indicate if it consists of aggregates of known proteins or if it constitutes a unique protein.

Isoaspartate has been found in the β-amyloid protein that comprises a significant portion of the protein aggregates in neuritic plaques and vascular amyloid deposits isolated from human brains afflicted with Alzheimer’s disease (25, 26). Unlike the proteins associated with these plaques, HMAP is highly soluble and is abundant in the brains of 7–8-week-old rats. Nevertheless, HMAP, like neuritic amyloid, may arise from the accumulation of proteins that are metabolically isolated, unable to be efficiently repaired by PIMT or to be broken down by intracellular proteases. Alternatively, HMAP may be a “normal” protein with highly unusual characteristics. Further studies on the structure, cellular localization, and developmental/age dependence of HMAP levels, should provide important clues to its significance and function.

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