Synthetic Peptide Substrates for the Erythrocyte Protein Carboxyl Methyltransferase

DETECTION OF A NEW SITE OF METHYLATION AT ISOMERIZED L-ASPARTYL RESIDUES

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Four hexapeptides of sequence L-Val-L-Tyr-L-Pro-(Asp)-Gly-L-Ala containing D- or L-aspartyl residues in normal or isopeptide linkages have been synthesized by the Merrifield solid-phase method as potential substrates of the erythrocyte protein carboxyl methyltransferase. This enzyme has been shown to catalyze the methylation of D-aspartyl residues in proteins in red blood cell membranes and cytosol. Using a new vapor-phase methanol diffusion assay, we have found that the normal hexapeptides containing either D- or L-aspartyl residues were not substrates for the human erythrocyte methyltransferase. On the other hand, the L-aspartyl isopeptide, in which the glycyl residue was linked in a peptide bond to the β-carboxyl group of the aspartyl residue, was a substrate for the enzyme with a $K_m$ of $6.3 \, \mu M$ and was methylated with a maximal velocity equal to that observed when ovalbumin was used as a methyl acceptor. The enzyme catalyzed the transfer of up to 0.8 mol of methyl groups/mol of this peptide. Of the four synthetic peptides, only the L-isohexapeptide competitively inhibits the methylation of ovalbumin by the erythrocyte enzyme. This peptide also acts as a substrate for both of the purified protein carboxyl methyltransferases I and II which have been previously isolated from bovine brain (Aswad, D. W., and Deight, E. A. (1983) J. Neurochem. 40, 1718–1726).

The L-isopaspartyl hexapeptide represents the first defined synthetic substrate for a eucaryotic protein carboxyl methyltransferase. These results demonstrate that these enzymes can not only catalyze the formation of methyl esters at the β-carboxyl groups of D-aspartyl residues but can also form esters at the α-carboxyl groups of isomerized l-aspartyl residues. The implications of these findings for the metabolism of modified proteins are discussed.

Eucaryotic protein carboxyl methyltransferases catalyze methyl ester formation on a wide variety of proteins both in vivo (1–3) and in vitro (1, 4–6). The methyl group incorporation is generally substoichiometric, however, and it appears that only a subpopulation of molecules in a given protein are capable of functioning as methyl acceptors (4–9). Based on our recent discovery of D-aspartic acid β-methyl ester in proteolytic digests of methylated erythrocyte proteins (3, 6, 10), we have postulated that one site of methylation is at aspartyl residues in the small fraction of proteins which have been racemized at that position to the uncommon D-configuration. Although the functional role of this modification is not known, we have proposed that the methylation reaction may play a role in the repair or degradation of age-modified proteins (10).

To further investigate this hypothesis, we needed a defined, homogeneous methyl-accepting substrate which could be stoichiometrically modified by the carboxyl methyltransferase. Since synthetic oligopeptide substrates have proven useful in studies of protein kinases (11–13), we decided to synthesize a homogeneous methyl-accepting substrate which could be stoichiometrically modified by the carboxyl methyltransferase. Such a defined substrate would be useful in delineating the chemical changes which may occur in the peptide backbone during the methylation and demethylation reactions.

Our original goal was to synthesize a hexapeptide containing 1 aspartyl residue in either the L- or the D-configuration. We expected that the peptide containing the D-aspartyl residue might be a substrate, whereas the corresponding L-peptide would not. We chose to study a modified sequence of human adrenocorticotropic hormone. This hormone has been shown to be a good, although substoichiometric, methyl-accepting substrate (7). Although it has been suggested that the site of methylation of this hormone is Glu-28 (14), alternative sites have not been ruled out. We were particularly interested in the possibility that a deamidated sequence containing an aspartyl residue at position 25 might instead be serving as the methyl-accepting site. In this respect, it has been shown that adrenocorticotropic hormone is readily deamidated at Asn-25, probably via a succinimide intermediate (15). This imide intermediate may be prone to racemization at the α-carbon (10), which would result in both D- and L-aspartyl residues at position 25. Alternatively, hydrolysis of the succinimide can produce an isomerized transpeptidation product where Gly-26 is linked via a peptide bond to the side-chain β-carboxyl rather than to the main-chain α-carboxyl of the newly formed aspartyl residue (15). Neither of these altered peptides produced by deamidation are prevalent in commercial preparations of adrenocorticotropic hormone, and carboxyl methyltransferase cannot be expected to be substoichiometric modifications as has been observed (7, 14). In light of these results, we thus synthesized...
the succinimide form of adrenocorticotropic hormone residues 22–27. We isolated the normal aspartyl (α-carboxyl) peptide and the isopaspartyl (β-carboxyl peptide) hexapeptide which were produced by mild-base hydrolysis of the succinimide (Fig. 1). We then compared the ability of the L- and D-aspartyl forms of these peptides to serve as substrates of the erythrocyte methyltransferase. Although neither the L-nor the D-normal peptides were stoichiometric methyl acceptors, we did find that the L-isopeptide was an excellent substrate. Thus, we have identified a new site of enzymatic carboxyl methylation. As with D-aspartyl residues, L-isopaspartyl residues in cellular proteins probably originate from the spontaneous degradation of L-aspartyl and L-asparaginyl residues, and their methylation may also be a signal for their repair or further metabolism (10).

While these studies were in progress, we became aware of parallel work by Aswad (16) in which he purified a deamidated subpopulation of native adrenocorticotropic hormone. He demonstrated that a deamidated, but not racemized, form was a high-affinity nearly stoichiometric methyl acceptor for the bovine brain protein carboxyl methyltransferases (16). Because he found inhibition of this reaction with the L-isopeptide described here, it is likely that both methylation reactions involve formation of a methyl ester on an α-carboxyl group of an isopaspartyl residue corresponding to position 25 on the intact adrenocorticotropic hormone molecule.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**

Peptides were synthesized manually using standard solid-phase procedures (17) with recent modifications (18). t-BOC1 amino acid derivatives were purchased from Vega Biochemicals (Tucson, AZ) except for t-BOC-L-Asp-β-OBzl ester which was obtained from Sigma and t-BOC-D-Asp-β-OBzl ester from Chemical Dynamics (South Plainfield, NJ). The stereochromatic purity of these latter two compounds was determined after removal of the protecting groups by refluxing in HCl/peroxide-free dioxane (1:1) for 24 h. The HCl/dioxane was then removed under reduced pressure, and the residue was reacted with L-leucine N-carboxyanhydride. The resulting diastereomeric dipeptides were separated and quantitated by amino acid analysis as described by Manning and Moore (Ref. 19; cf. Ref. 10). Each aspartic acid derivative was found to contain less than 1% of the contaminating enantiomer.

The first amino acid was coupled to the chloromethylated polystyrene resin (1.62 g, 1.26 meq/g, 1% cross-linked, Bio-Rad) in refluxing dimethylformamide (99%, Aldrich) and neutralized stepwise (one wash of 1:9 triethylammonium acetate, 0.2 M NaCl, followed by a 10-min reaction period). The crude peptide, obtained by lyophilization, was dissolved in water to give a solution was washed from the resin, and the solvent was removed by evaporation under reduced pressure at 50 °C. The resulting oil was dissolved in methanol and methanol:water (1:1) and evaporated several times to remove traces of HBr. This oil was then dissolved in 10% acetic acid and reacted with Coomassie brilliant blue until the aqueous phase was no longer yellow. The crude peptide, obtained by lyophilizing the aqueous phase, consisted largely of the succinimide derivative (see below; cf. Ref. 23).

**Purification and Isolation of Peptides**

Peptides were purified by C18 reverse-phase HPLC. The system consisted of two Waters M-45 pumps, a M-660 solvent programmer, a UK sample injector and a model 441 ultraviolet detector. Peptides were eluted with a gradient of acetonitrile in trifluoroacetic acid similar to that described previously (24, 25). In all cases, columns were equilibrated with the initial solvent (A) which consisted of 0.1% trifluoroacetic acid in water. Solvent B was 90% acetonitrile, 10% water, and 0.1% trifluoroacetic acid. These solvents were prepared by dilution of a 10% stock (v/v) of Pierce Sequential grade trifluoroacetic acid which had been further purified by passage through a Waters Sep-Pak C18 sample preparation cartridge. Peptide purification was accomplished in two steps. First, the succinimide derivative of the peptide (Fig. 1) was purified from the crude peptide mixture (Fig. 2, left). Here, aliquots of samples were injected onto an Alltech Econosphere C8 column (4.6 × 250 mm, 5-μm spherical resin) and were eluted at 1 ml/min with a 40-min linear gradient of 0–40% solvent B (100–60% solvent A). Fractions (1 min) were collected and their absorption was measured at 274.5 nm. The major fraction eluting at 27.5–30 min was identified as the succinimide (see "Results") and was pooled and lyophilized. In a second step, the normal and isohexapeptides were obtained by resuspending the succinimide in water and mixing with an equal volume of 0.45 M sodium borate at pH 10.25 (the final pH was 10.10). This solution was incubated at 37 °C for 15 min to hydrolyze the inside. The hydrolysis was quenched by addition of 0.35 ml of trifluoroacetic acid. The hydrolysates were separated by HPLC (Fig. 2, right). Here, 0.5–1 mg of peptide was injected into the same column described above, but elution was now performed with a 30-min 10–40% gradient of solvent B using a concave gradient (curve 8 of the M-660 solvent programmer). Fractions (1 min) were collected and analyzed as described above.

**Characterization of Peptides**

**Thin-layer Electrophoresis**—The electrophoretic mobility of the succinimide and the normal and isohexapeptides was measured to confirm the identity of these products. Sample (6 nmol) was applied to the middle of a 20-cm Eastman cellulose thin-layer sheet along with standards of aspartic acid, glutamic acid, lysine, alanine, and glycine. Electrophoresis was performed in a buffer of 2% pyridine, 0.95% acetic acid (pH 5.2) at 20 V/cm for 30 min. Peptides and amino acids were detected by ninhydrin spray. The succinimide peptides had mobilities equal to that of the neutral amino acid standards; the normal and isohexapeptides were acidic.

**Amino Acid Analysis and Proteolytic Digestion**—Peptides were acid hydrolyzed in 6 M HCl in vacuo at 108 °C for 24 h. Mepacartic acid (0.1%) was added to protect tyrosine from oxidative degradation. Hydrolysates were evaporated under reduced pressure at 60 °C and were resuspended in amino acid analyzer sample buffer (pH 2.2, sodium citrate, 0.2 M in Na+; Pierce Chemical Co.). Peptides were digested with proteolytic enzymes under two conditions to establish the identity of the normal and isohexapeptides (26). The first procedure utilized a mixture of cytosolic leucine aminopeptidase (porcine kidney, Sigma type III-CP, 100 units/mg of protein) and prolidase (porcine kidney, Sigma, 200 units/mg of protein; and isohexapeptides were obtained by resuspending the succinimide and 150 μg of prolidase) were mixed and collected by centrifugation for 4 min at 8000 × g. The pellet was resuspended in 0.25 ml of 8.8 M MgSO4, 88.5 mM Tris-HCl (pH 8.0), and 106 mM NaCl. An equal volume of peptide (20 μl, 30 nmol) and protease preparation (20 μl) was mixed and incubated at 37 °C for 1 h. The reaction was quenched with 10 μl of 6 M HCl and 0.25 ml of 2 M NaCl, 0.2 ml sample buffer (see above). The second enzymatic digestion procedure utilized yeast carboxypeptidase Y (bakers yeast, Sigma, 100 units/mg of protein). The lyophilized enzyme was dissolved in water to give

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1 The abbreviations used are: t-BOC, N-t-butyloxycarbonyl; Bzl, benzyl; HPLC, high-performance liquid chromatography; AdoMe, S-adenosyl-L-methionine; [3H]AdoMet, S-adenosyl-L-[methyl-3H]methionine.
a protein concentration of 4 mg/ml and about 16 mg/ml of pH 5 sodium citrate buffer. Peptide (30 nmol) and carboxypeptidase Y (60 µg of protein) were incubated in a buffer of 66.7 mM sodium citrate (pH 6.0) in a final volume of 60 µl for 24 h at 37 °C. These digestions were quenched as described above.

All amino acid analyses were performed on a Beckman Model 120C automatic amino acid analyzer. Amino acid eluates were eluted from a column (0.9 cm x 54 cm) of PA-28 resin at 56 °C at a flow rate of 1.2 ml/min. Elution was performed with pH 3.25 sodium citrate buffer (0.2 M in Na⁺, Pierce Chemical Co.) for 100 min followed by pH 4.25 sodium citrate buffer (0.2 M in Na⁺). Basic amino acids were analyzed on a column (0.4 cm x 24 cm) with a 3.8 cm sodium citrate buffer (0.38 M in Na⁺). The analyzer was equipped with high-sensitivity cuvettes and recorder so that about 10 nmol of amino acid would give a full-scale ninhydrin response. Amino acids were quantified with respect to an amino acid standard (Pierce Standard H). 

Analysis of D-/L-Aspartic Acid Ratios—Peptides (10 nmol) were hydrolyzed in 6 M HCl in vacuo at 108 °C for 6 h. The hydrolysates were dried under reduced pressure at 60 °C and then resuspended in 3 ml of water and dried down again. These latter steps were repeated two more times to remove the last traces of HCl. The final residue was dissolved in 0.5 ml of 1 M acetic acid and applied to a column (0.7 x 30 cm) of Dowex AG 1-X8 resin equilibrated with 150 mM acetic acid. All of the amino acids except aspartic acid were eluted from the column in a 15-ml wash. The aspartic acid fraction could be eluted from the column in 8 ml of 1 M acetic acid and lyophilized. The D/L composition of this material was quantitated by the method of Aswad (28) which involves the separation by HPLC of the two isomeric derivatives of D- and L-aspartic acid present in a racemic reaction with o-phthalaldehyde and N-acetyl-L-cysteine. The fluorescent aspartic acid derivatives were applied to a Waters Resolve C₅ column (0.5 µm, 3.9 x 150 mm) equilibrated and eluted with 46.8 mM sodium acetate (pH 5.80) and 3.2% methanol. The derivatives were detected using a Gilson Spectra-Glo Flurometer equipped with a 350-380 nm excitation filter, a 430-600 nm emission filter, and a 15-µl flow cell. The amount of D- and L-isomers was determined from the relative peak heights after correction for the slightly different fluorescent intensities of the two diastereomeric derivatives found using a racemic mixture of aspartic acid.

Enzymatic Methylation Reactions

Preparation of Erythrocyte Cytosol As a Source of Carboxyl Methytransferase—A cytosolic fraction was prepared from human red blood cells on the same day that the blood was drawn from healthy volunteers into heparinized vacutainers. The plasma and buffy coat were removed from the membrane pellet and stored in small aliquots at −20 °C. The protein concentration of this cytosol was determined from the absorbance of diluted fractions at 280 nm using an extinction coefficient of 2.4 for a 1 mg/ml solution (29).

Preparation of [3H]AdoMet and Specific Activity Determination—A concentrated stock solution of [3H]AdoMet was prepared by mixing 1 volume of 6.3 M nonisotopically labeled S-adenosyl-L-methionine (Boehringer-Mannheim, HSCG salt, 98% in 3-5um concentration determined by the absorption at 290 nm of diluted samples using a molar extinction coefficient of 15,400 (30)) in 10 mM HCl with 1 volume of S-adenosyl-L-[methyl-3H]methionine (Amersham Corp., 15 Ci/mmol, 66.7 µM in dilute H₂SO₄ (pH 2.5-3.5) and diluting with 8 volumes of 10 mM HCl. The purity of this preparation was analyzed by thin-layer chromatography as described (31). The specific activity of the [3H]AdoMet was determined from the radioactivity and the UV absorbance of the major peak eluting at 25–29 min. A minor peak containing about 25% of the UV material and 10% of the radioactivity eluting at 31–35 min was not identified but may represent the (R)-diastereomer of AdoMet (32). These two peaks accounted for more than 90% of the total radioactivity and UV-absorbing material.

Methytransferase Reaction Mixtures—Peptides were incubated with [3H]AdoMet and enzymes under conditions similar to those described by McFadden et al. (33). [3H]AdoMet was prepared by mixing 1 volume of the isotopically diluted stock solution described above in 6.25 volumes of 0.4 M sodium citrate buffer (pH 5.5) and 2.75 volume of water. The enzymatic reaction mixture included 20 µl of this buffered [3H]AdoMet preparation, 30 µl of erythrocyte cytosol (about 40 mg of protein/ml, see above), 40 µl of assay buffer (pH 6.0, 0.2 M sodium citrate, 0.2 M NaCl, 5 mM sodium phosphate (pH 7.4), 0.1% bovine serum albumin, 0.001% sodium azide, 10% glycerol and 1 µM [carboxyl methyl] peptide substrate. The final pH of the incubation mixture was found to be 6.0. The peptides were made up in water, and their concentration was determined by the absorbance of a stock solution at 274.5 nm using an extinction coefficient of 1402 M⁻¹ cm⁻¹ for the tyrosine residues. In some experiments, purified bovine brain protein carboxyl methyltransferase diluted in lysis buffer was used instead of cell cytosol as a source of methyltransferase. Control experiments were performed routinely in which ovalbumin (chicken egg, crystallized and lyophilized, Sigma grade V) was used as a substrate. In these incubation mixtures, 10 µl of water and 40 µl of 80 mg/ml of ovalbumin in assay buffer were added in place of the peptide and assay buffer, respectively. In all cases, the final AdoMet concentration was calculated to be 7.3 µM.

Incubations were performed at 37 °C. In experiments where initial velocities were measured, reactions were quenched immediately after 30 s (0.5 min, 20 min and 20 min of incubation). Trypan blue incorporation of methyl groups was shown to be linear under these conditions. Specific quench procedures are described below. All assays were performed in duplicate.

Methanol Vapor Diffusion Assay for Protein Carboxyl Methyltransferase

Quantitation of Methyl Esters by Vapor Diffusion Assay—The principle of this assay is that [3H]methanol released from base-treated protein and peptide [3H]methyl esters can diffuse from an aqueous reaction mixture into a volume of liquid scintillation fluid, whereas the [3H]AdoMet substrate and the bulk of its degradation products cannot. Briefly, the assay consists of placing an open tube containing base-treated protein carboxyl methyltransferase reaction mixtures (see above) inside a capped tube which is placed in a chamber containing scintillation fluid. After 24 h at room temperature, the vial is opened to remove the insert tube and the vial is then closed and counted.

Specifically, 80 µl of the 100-µl methyl methanethiol reaction mixture is added at the end of the incubation period to 80 µl of a quench solution (0.6 M sodium borate, 1% sodium dodecyl sulfate (pH 10.2) in a 400-µl polypropylene microfuge tube (4.4 mm inner diameter, × 44 mm). This tube is capped until the start of the diffusion procedure. At this time, control vapor diffusion tubes are also prepared which contain 5 µl of purified [3H]methanol (see below) and all of the buffer and other components of the enzyme reaction mixture except the peptide substrate. After 24 h at room temperature, the vial is opened to remove the microfuge tube and the vial is then closed and counted.

The efficiency of the methanol transfer is calculated from the radioactivity obtained when a [3H]methanol standard is placed in the microfuge tube in the assay above and that when 4 µl of the [3H]methanol standard are counted directly in 2.4 ml of the ACS II fluor. This efficiency is approximately 30-40% under these conditions. Using ovalbumin as a substrate, a background (determined for samples without enzyme or methyl acceptor or for zero time incubations) equivalent to 3-5 pmol and a signal (complete enzyme reaction mixture) of 30-90 pmol are typical.

Preparation of a [3H]Methanol Standard—We found it necessary to purify commercial samples of [3H]methanol for use in this assay. Isotope (20 nmol, 50 mCi/mmol, New England Nuclear) was diluted with 1 ml of nonisotopically labeled methanol and 3 ml of water. Fractional distillation was performed using a 15-cm Vigreaux distillation head, and material distilling between 64.5 and 65 °C was collected. The final specific activity of this material was about 300 cpm/µl. The behavior of this purified [3H]methanol in the vapor phase was similar to that of a [3H]methanol preparation, kindly provided by Dr. Dana Asawd (Department of Psychology, University of California, Irvine), and purified by the same procedure. However, it was found that the percentage of the isotopes which were transferred in the assay at a given time differed by a
hexapeptides used in these studies.

In experiments designed to selectively detect the methylation of ovalbumin in an incubation mixture which also contained peptides, the vapor-phase assay described above was not used. Here, methyltransferase reaction mixtures were prepared as above except that 40 \( \mu \)l of ovalbumin in assay buffer replaced the equivalent volume of assay buffer alone. At the end of the incubation at 37 °C, 80 \( \mu \)l of the 100-\( \mu \)l reaction mixture were spotted on filter paper and processed as described (38) for trichloroacetic acid-precipitable radioactivity. Control experiments demonstrated that the methylated hexapeptides were not precipitated under these conditions, even in the presence of carrier ovalbumin.

RESULTS

Characterization of Synthetic Normal and Isohexapeptides—Hexapeptides corresponding to the sequences L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala and L-Val-L-Tyr-L-Pro-D-Asp-Gly-L-Ala were synthesized by solid-phase methods as described under "Experimental Procedures." After cleavage from the resin, HPLC analysis revealed a major UV-absorbing species and several minor components (Fig. 2, left). Since this peptide contained an aspartylglycyl sequence, we expected the major product would correspond to the succinimide (free \( \beta \)-carboxyl group on Asp, \( \beta \)-peptide linkage) and because there was no release of these amino acids in parallel carboxypeptidase Y digestions, the neutral electrophoretic mobility of the product at pH 5.2 (see "Experimental Procedures"), the generation of two hydrolysis products with acidic electrophoretic mobilities at pH 5.2, and the 220-MHz NMR spectrum (data not shown).

The normal and isohexapeptides (see Fig. 1 for structures) were prepared from the HPLC-purified succinimide by mild-base hydrolysis and were separated by HPLC (Fig. 2, right). The purity of each of these compounds was shown by analytical HPLC (Fig. 3). Because previous studies had indicated that the isopeptide would be the predominant product of such a hydrolysis (23, 37, 38), we tentatively assigned the fraction which eluted first from the HPLC and contained approximately 70% of the material as the isopeptide for both the D-aspartyl and the L-aspartyl peptide preparations. The second peak was then assigned as the normal peptide. Although the data shown in Fig. 2 are for the L-hexapeptide synthesis, both the \( \gamma \)- and the D-succinimides and their hydrolysis products exhibited very similar elution characteristics (cf. Fig. 3). These assignments were confirmed by the data presented in Tables I and II as well as by direct sequence studies (see below).

The amino acid compositions of the peptides are shown in Table I. For each of the four peptides synthesized, a stoichiometric number of each of the amino acid residues was obtained and no other amino acids were detected. Since the peptides were initially obtained in the succinimide form and because this imide may be racemization prone (10), the D/L content of the aspartyl residue of each peptide was also examined (Table I). In no case was the content of the contaminating enantiomer greater than 10.0%.

Proteolytic digestion of each of the four hexapeptides was used to verify the assignments of the normal and iso-configuration of the HPLC-purified peptides. It has been shown that the isoaspartyl \( \beta \)-peptide bond is not cleaved by proteolytic enzymes such as leucine aminopeptidase and prolidase (26). We reasoned, therefore, that such an enzymatic digestion of the L-normal hexapeptide should be complete and result in the formation of each of the six free amino acids. On the other hand, only valine, tyrosine, and proline would be released as free amino acids in the L-isopeptide digest, and the remaining tripeptide would be intact. This was in fact the experimental result obtained and these data are shown in Table II. Although we have not identified the tripeptide product of this digestion, we have found that a new ninhydrin-positive peak elutes on amino acid analysis close to the expected position of L-aspartyl-\( \beta \)-glycyl-L-alanine (cf. Ref. 40).

A similar set of results were obtained when carboxypeptidase Y was used to digest the L-peptides (Table II). Here, complete digestion was obtained for the L-normal hexapeptide but only the first 3 residues were released from the L-isopeptidase digestion. In this case, the enzyme appears to have recognized the L-aspartyl-\( \beta \)-glycyl-L-alanine portion of the hexapeptide as a single C-terminal amino acid (the free \( \alpha \)-carboxyl is on the aspartyl residue) and releases this as an intact unit after cleaving the prolylaspartyl peptide linkage.

Proteolytic digestion was also performed with the D-aspartyl hexapeptides. No digestion was found with either the D-normal or the D-isohexapeptide with carboxypeptidase Y since the release of small amounts of aspartic acid, glycine, and alanine from the L-isohexapeptide with leucine aminopeptidase and prolidase can be explained in two ways. First, there may be small amounts of contaminating protease(s) capable of hydrolyzing isopeptide linkages. Second, these enzymes may themselves be capable of slow cleavage of these bonds. It does not appear that these free amino acids originate from contaminating normal L-hexapeptide because there is no peak of the normal peptide in the purified isopeptide material by HPLC analysis (Fig. 3) and because there was no release of these amino acids in parallel carboxypeptidase Y digestions.
Protein Carboxyl Methylation at L-Isoaspartyl Residues

Fig. 2. Purification of synthetic hexapeptides by HPLC. The details of the preparative HPLC systems and the procedure for base hydrolysis of the succinimide are given under “Experimental Procedures.” Left, isolation of hexapeptide imide from the crude L-peptide obtained by solid-phase synthesis. Right, isolation of the iso- and normal hexapeptide after mild-base treatment of the L-imide. Note that the elution conditions used here are different from those used to purify the succinimide (left). In this latter HPLC system, the small amount of unhydrolyzed succinimide elutes after the normal hexapeptide at 34 min. Similar results to those obtained above were observed for the purification of the D-hexapeptides.

Fig. 3. Analytical HPLC of the four purified synthetic hexapeptides. In each chromatogram, about 4 µg of hexapeptide in 0.1% trifluoroacetic acid were analyzed using the Econosphere C18 column and the elution gradient used to isolate the succinimide (Fig. 2, left; cf. “Experimental Procedures”). The elution time is shown above each peak. The arrow near the base-line in each panel indicates the expected elution position of the isopeptide for the D- and L-normal peptides and that of the normal peptide for the D- and L-isopeptides. The peaks eluting between 3 and 5 min are injection artifacts.

D-amino acid-containing peptides are competitive inhibitors of this enzyme and are cleaved slowly if at all (41). Digestion of both D-hexapeptides with leucine aminopeptidase and prolidase resulted in the release of valine, tyrosine, proline, and a fourth compound. Since these proteolytic enzymes would not be expected to cleave bonds past the D-aspartyl residue, even if it were in a normal peptide linkage, the fourth compound in both cases would be a tripeptide. For the D-isohexapeptide, the tripeptide expected is D-aspartyl-β-glycyl-L-alanine and for the D-normal hexapeptide, the tripeptide would be D-aspartylglycyl-L-alanine. Ninhydrin-positive material corresponding to each of these compounds has been identified in these digests (Table II; cf. Ref. 40).

Finally, we subjected both the L-normal and the D-normal peptides to automated gas-phase sequencing (42). These studies, performed by Dr. Audree Fowler of the UCLA Protein Microsequencing Laboratory, confirmed both the sequence of these peptides and the absence of any isopeptide linkages.

Methanol Vapor Diffusion Assay of Protein Carboxyl Methyltransferase with Peptide Methyl-accepting Substrates—Previously described assays for protein carboxyl methyltransferase generally involve the physical separation of the radiolabeled methyl donor ([3H]AdoMet) from the radiolabeled protein product by precipitation of the macromolecules with
a distillation assay relatively tedious, and there is often a large background of labeled AdoMet substrate. A different type of assay has recently been described in which radiolabeled methanol is separated from AdoMet by vapor-phase diffusion under relatively mild conditions (46). In this procedure, an open tube containing a base-treated incubation mixture is placed inside a larger vial containing unlabeled methanol. After further incubation, the radiolabeled methanol (but not the radiolabeled AdoMet) was found to equilibrate into the methanol phase. We found, however, that we could not obtain the efficient transfer of methanol described previously (46). We were able to modify this assay by replacing methanol in the outer vial with aqueous liquid scintillation fluid and have developed a useful assay (see "Experimental Procedures") which should be generally applicable to assays of carboxyl methylation of macromolecular as well as peptide methyl acceptors.

**Synthetic Hexapeptides As Methyl Acceptors for the Erythrocyte Protein Carboxyl Methyltransferase**—The ability of each of the four synthetic hexapeptides to function as methyl acceptors in a reaction with protein carboxyl methyltransferase from erythrocyte cytosol and [3H]AdoMet was studied. In initial velocity experiments in which the amount of each peptide was varied over a range of 0.5-25 μM, neither the D-nor the L-normal hexapeptide displayed methyl-acceptor activity (Fig. 4). On the other hand, the L-isoaspartyl hexapeptide was shown to be an effective substrate for the methyltransferase activity. The D-isoaspartyl hexapeptide preparation also displayed methyl-acceptor activity, but this activity was much lower than that of the L-isoaspartyl peptide preparation and could in fact be quantitatively accounted for by the presence of an L-isoaspartyl impurity. Michaelis constants of the carboxyl methyltransferase for the L-isohepta-

![Image](https://example.com/image.png)

**Fig. 4.** Initial velocities of the erythrocyte cytosolic protein carboxyl methyltransferase using purified hexapeptides as methyl acceptors. Methyltransferase activity was measured by the vapor diffusion assay described under "Experimental Procedures." Each point shown represents an average of duplicate determinations. Background values obtained for parallel incubations without substrates have been subtracted. Lines drawn for the D-isoheptapeptide (Iso-D), the normal L-hexapeptide (L) and the normal D-hexapeptide (D) were obtained using a least-squares linear regression fit of the data. The curve drawn for the L-isoheptapeptide (Iso-L) was calculated using the Michaelis constants derived from Fig. 5.

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**Table I**

| Amino acid composition and D-/L-Asp acid ratio of synthetic hexapeptides | D-/L-Asp acid ratio |
|-----------------------------|-------------------|
| **Amino acid composition** | **Val** | **Tyr** | **Pro** | **Asp** | **Gly** | **Ala** |
| L-Hexapeptide               | 1.10 | 0.98 | 0.85 | 1.21 | 1.34 | 1.00 | 0.033 |
| L-Isohexapeptide            | 0.98 | 0.85 | 0.87 | 0.96 | 1.07 | 1.00 | 0.098 |
| D-Hexapeptide               | 1.05 | 0.99 | 1.05 | 1.07 | 1.09 | 1.00 | 33.2  |
| D-Isohexapeptide            | 0.97 | 0.86 | 1.06 | 1.00 | 1.17 | 1.00 | 8.95  |

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**Table II**

| Hexapeptide | Protease(s) | Free amino acid | Dipeptide | Tripeptide |
|-------------|-------------|-----------------|-----------|------------|
| L-Hexapeptide | CPY         | Val | Tyr | Pro | Asp | Gly | Ala | 0.81 | 0.70 | 0.62 | 0.77 | 0.55 | 0.00 |
| L-Isohexapeptide | LAP/Prol | Val | Tyr | Pro | Asp | Gly | Ala | 1.16 | 1.16 | 0.83 | 1.00 | 1.11 | 1.03 |
| D-Hexapeptide | CPY         | Val | Tyr | Pro | Asp | Gly | Ala | 0.73 | 0.70 | 0.80 | 0.00 | 0.00 | 0.50 |
| D-Isohexapeptide | LAP/Prol | Val | Tyr | Pro | Asp | Gly | Ala | 1.50 | 1.41 | 1.22 | 0.26 | 0.20 | 0.19 |
| D-Hexapeptide | CPY         | Val | Tyr | Pro | Asp | Gly | Ala | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| D-Isohexapeptide | LAP/Prol | Val | Tyr | Pro | Asp | Gly | Ala | 1.05 | 1.00 | 0.84 | 0.00 | 0.00 | 0.00 |

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* Determined by standard amino acid analysis as described under "Experimental Procedures." Results are expressed as a molar ratio with respect to the first residue attached to the solid-phase support.

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All other amino acids were present at levels of less than 0.05 mol/mol of Ala.

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*Performed by the method of Aswad (28). No correction has been made for the small amount of racemization that occurred during hydrolysis.

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trichloroacetic acid or other denaturants. Since the hexapeptides synthesized here were not likely to be precipitated by such methods, we sought an assay in which such separation methods were not required. Protein and peptide methyl esters can be rapidly hydrolyzed in base to produce methanol (43), and procedures have been described to assay methyl transfer reactions by quantitating the amount of methanol formed in a distillation assay (44, 45). However, these methods are relatively tedious, and there is often a large background of radioactivity from the decomposition products of the radiolabeled AdoMet substrate. A different type of assay has recently been described in which radiolabeled methanol is separated from AdoMet by vapor-phase diffusion under relatively mild conditions (46). In this procedure, an open tube containing a base-treated incubation mixture is placed inside a larger vial containing unlabeled methanol. After further incubation, the radiolabeled methanol (but not the radiolabeled AdoMet) was found to equilibrate into the methanol phase. We found, however, that we could not obtain the efficient transfer of methanol described previously (46). We were able to modify this assay by replacing methanol in the outer vial with aqueous liquid scintillation fluid and have developed a useful assay (see "Experimental Procedures") which should be generally applicable to assays of carboxyl methylation of macromolecular as well as peptide methyl acceptors.
peptides to inhibit the incorporation of methyl esters into ovalbumin methylation, and this inhibition was seen only at ovalbumin (Table III). Only the L-isohexapeptide inhibited a protein substrate for the eucaryotic carboxyl methyltransferase (1, 33). To determine the relationship between the shown here, we measured the ability of each of the four methyltransferase.

Near-Stoichiometric Methylation of the L-Isohexapeptide—When low concentrations of the L-isohexapeptide are incubated with erythrocyte cytosol and [3H]AdoMet, nearly stoichiometric levels of methyl group incorporation are observed after 3 h at 37 °C (Fig. 6). Maximal levels of methylation obtained in various experiments have ranged from 0.55 to 0.80 mol of methyl group/mol of L-isohexapeptide preparation. These 3-h values approach the theoretical value of 0.91 for the fraction of the isohexapeptide in the L-configuration (Table I). These results and those of Aswad (16) with deamidated adrenocorticotropic hormone as a substrate represent the first examples of nearly stoichiometric methylation of a defined peptide or protein by the eucaryotic protein carboxyl methyltransferase.

Inhibition of Carboxyl Methylation of Ovalbumin by Synthetic Hexapeptides—Ovalbumin has been frequently used as a protein substrate for the eucaryotic carboxyl methyltransferase (1, 33). To determine the relationship between the ovalbumin methylation reaction and the peptide methylation shown here, we measured the ability of each of the four peptides to inhibit the incorporation of methyl esters into ovalbumin (Table III). Only the L-isohexapeptide inhibited ovalbumin methylation, and this inhibition was seen only at low concentrations of ovalbumin near the K_m value of the methyltransferase for ovalbumin. Although it was not possible to monitor peptide methylation in the same reaction mixtures, the results suggest that the L-isohexapeptide is functioning as a competitive inhibitor of ovalbumin methylation by binding to the methyltransferase itself. Furthermore, because no inhibition was detected with the other three peptides, these compounds do not appear to tightly bind the methyltransferase either as substrates or inhibitors.

Synthetic Hexapeptides As Substrates for the Purified Bovine Brain Methyltransferase Isozymes—It has been shown that a deamidated form of adrenocorticotropic hormone, which contains only small amounts of racemized D-aspartyl residues, is a high-affinity methyl group acceptor for both of the two purified isozymes of the bovine brain protein carboxyl methyltransferase (16). We tested here the ability of the four synthetic peptides to serve as methyl group acceptors for these purified enzymes, supplied to us by Dr. Aswad (Table IV). As with the erythrocyte cytosolic enzyme(s), the peptide with the highest methylation when reacted with the purified brain isozymes is the L-isohexapeptide. Little or no
concentration was 32 mg/ml.

Methylation of peptide and protein substrates by erythrocyte and brain protein carboxyl methyltransfemse

| Substrate               | Human red cell cytosol | Purified bovine brain isozyme I | Purified bovine brain isozyme II |
|-------------------------|------------------------|---------------------------------|---------------------------------|
| 1-Hexapeptide           | 0.90                   | 470                             | 500                             |
| 1-Isohexapeptide        | 9.88                   | 10,700                          | 7,620                           |
| D-Hexapeptide           | -0.32<sup>a</sup>      | -236<sup>c</sup>               | 50                              |
| D-Isohexapeptide        | 2.59                   | 1,990                           | 1,300                           |
| Ovalbumin               | 7.76                   | 14,900                          | 12,900                          |

<sup>a</sup>The hexapeptide concentration was 20 μM, and the ovalbumin concentration was 32 mg/ml.

<sup>c</sup>Methyltransferase activity was assayed by the vapor diffusion assay as described under "Experimental Procedures." Average values for duplicate determinations are shown. Background activities obtained in parallel incubations without substrate have been subtracted.

<sup>a</sup>Enzymes were obtained from Dr. Dana Aswad (University of California, Irvine). When assayed using γ-globulin as a methyl acceptor at 30 °C (4), the activity of protein carboxyl methyltransferase I was 16,900 pmol/min·mg of protein, and protein carboxyl methyltransferase II had an activity of 12,000 pmol/min·mg of protein.

<sup>c</sup>Values obtained for these samples were below those of the background without substrate.

incorporation was detected with the other three synthetic peptides. These results support the conclusion of Aswad (16) that the brain enzymes catalyze the transfer of methyl groups to α-carboxyl groups on L-aspartyl residues involved in isopeptide linkages.

**DISCUSSION**

Both D-Aspartyl and L-Isoaspartyl Residues Are Sites of Methylation of Eucaryotic Methyltransferases—Many proteins have been identified as methyl-accepting substrates of eucaryotic protein carboxyl methyltransferases (for reviews, see Refs. 1, 9, and 47). It now appears that most, if not all, cellular proteins can be carboxyl methylated to some extent. Generally, the extent of modification is very small (often less than one methyl group/1000 polypeptide chains), suggesting that only a subpopulation of native proteins function as substrates (2–8). Previous results have indicated that one site of methylation was likely to be on modified aspartyl residues in which the amino acid was in the D-configuration (3, 6, 10). The results of the present study indicate that eucaryotic protein carboxyl methyltransferases can also catalyze methylation on a second type of modified aspartyl residue. Here, the amino acid is in the L-configuration, but the carboxyl peptide bond is made via the side-chain β-carboxyl group rather than the α-carboxyl group. Both of these types of modified residues are presumably formed from L-aspartyl and/or L-asparaginyl residues by racemization and transpeptidation reactions (Fig. 7). These reactions appear to be consequences of nonenzymatic degradative or aging processes. Succinimidies formed by deamidation reactions of asparagine residues are likely intermediates to explain the appearance of aspartyl isopeptide sequences (48), although other origins are possible. In the present study the isoaspartyl sequences were formed by mild-base treatment of the succinnimides formed during the cleavage of the peptide from the resin, there is evidence that these sequences can also form under physiological conditions in proteins. For example, isomerized aspartyl peptides have been detected in enzymatic digests of proteins (38, 50, 51), and significant amounts of aspartyl β-peptides are found in urine. The latter compounds are thought to be natural proteolytic products of isomerized proteins (52–54).

The specificity of eucaryotic protein carboxyl methyltransferases for aspartyl derivatives (rather than L-aspartyl residues) is supported by studies which show that heating and/or base treatment increases the methyl-accepting ability of several proteins. For example, one of the best methyl-acceptor substrates is a base-treated fraction of calf thymus protein (F-P-100; Ref. 56). Other studies have shown that heating and/or base treatment can effect a 10-fold increase in the ability of calmodulin to be a methyl acceptor (57) or a 6–50-

<sup>a</sup>It is also possible that L-isooaspartyl residues in proteins may be derived from ribosomal synthesis where the tRNA is erroneously attached to t-aspartic acid via the β-carboxyl group rather than the α-carboxyl group. It is significant to note that carboxyl methylation has been detected on polysome-attached newly synthesized proteins (49).

<sup>c</sup>The extent of racemization of the imide is not clear. From the synthetic studies reported here, the amount of racemization in the final hexapeptide products due to imide racemization would be less than 10%.

<sup>a</sup>It should be noted that evidence has also been presented to suggest that at least a fraction of the urinary dipeptides may be a product of a reaction between free asparagine and glycine (55).

<sup>a</sup>L. S. Brunauer and S. Clarke, unpublished observations.
fold increase in that of ribonuclease (cf. Ref. 58). At this
time, there is no evidence that D-glutamyl or L-isoglutamyl
residues are substrates for protein carboxyl methyltransfer-
ases. It should also be noted that there is no definitive
evidence yet for the methylation of L-aspartyl or L-glutamyl
residues in eucaryotic systems, although L-glutamyl methyl
esters are the product of a bacterial carboxyl methyltrans-
ferase (59, 60). It does seem unlikely, however, based on the wide
variety of proteins that serve as methyl acceptors and from
the stoichiometric incorporation of methyl groups into these
proteins that the eucaryotic methyltransferases presently
isolated is able to catalyze the formation of methyl esters on unmodified L-aspartyl and L-glutamyl residues (9, 61).

How Many Eucaryotic Protein Carboxyl Methyltransferases
Exist?—Isozymes of protein carboxyl methyltransferase have
been identified in many tissues (62), and two isozymic forms
have been purified and characterized in cow brain by Aswad
and Deight (4). Since we have now identified two methyltrans-
ferase reactions (at D-aspartyl and L-isoaspartyl sites), these
isozymes may be thought to catalyze different reactions. The
results presented here (Table III) and by Aswad (16) suggest
that this is not the case because both enzymes catalyze methyl
ester formation with the L-isohexapeptide, but neither is
able to catalyze methylating the D-normal hexapeptide under the
conditions used here. In addition, we have shown that both
of the purified brain methyltransferases catalyze the forma-
tion of L-aspartyl beta-methyl ester when each enzyme is incu-
bated with red cell membranes as a methyl-accepting sub-
strate.10

It is possible, in fact, that a single enzyme might recognize
both D-aspartyl and L-isoaspartyl residues because the stereo-
configuration of these residues is similar. When the polypep-
tide chain is held in a fixed position, both carboxyl groups are
oriented in the same direction, and the only difference is in the
position of one methylene group (Fig. 8). The failure of the D-aspartyl hexapeptide synthesized here to act as an
effective methyl acceptor in the present study may reflect
additional requirements for specific amino acid sequences and/or
three-dimensional structures in D-aspartyl substrates. Of course,
it is also possible that D-aspartyl methylation is catalyzed by a separate methyltransferase which has not yet
been identified. We consider this unlikely, however, since the
crude cytosolic fraction of erythrocytes, which should contain
all of the soluble methyltransferase species of the cell, shows
the same specificity for the peptide substrates as do the purified enzymes.

Physiological Methylation at D-Aspartyl and L-Isospartyl
Sites—Up to the present time, we have been able to detect
only methylation at D- aspartyl sites in erythrocyte proteins
labeled with radioactive methyl groups in intact cells (3, 10,
61) or in membrane fractions incubated with purified methyl-
transferase preparations (6, 61). In each of these experi-
ments, proteolytic digestions using carboxypeptidase Y have
been employed to isolate radiolabeled D-aspartic acid beta-
methyl ester from the methylated polypeptides. The yields of
this product are generally low, and we have attributed this to
the hydrolysis of methyl esters during the slow cleavage of the
peptide bonds surrounding the D-aspartyl residue (61).
However, it is possible that other types of esters are present,
and we have thus searched for evidence of L-isospartyl
methylation in similar proteolytic digests of 3H-methylated
proteins. We would expect that protease treatment would
result in the formation of L-aspartic acid alpha-aspartyl methyl
ester or of an isospartyl peptide such as L-aspartyl alpha-methyl
ester-beta-glycine. We have synthesized the latter compound as well as L-aspartic acid alpha-methyl ester. Both compounds were found to elute near the position of leucine on amino acid
analysis and were well separated from aspartic acid beta-methyl
ester. However, we have not found evidence for the formation of radioactive compounds from proteolytic digests of 3H-
methylated erythrocyte proteins which co-migrate with stand-
ards of these synthetic alpha-carboxyl methyl esters on amino
acid analysis (data not shown). There are several possible
explanations for these negative results. Although the hydroly-
sis of neither of the synthetic alpha-methyl ester standards was
found to be catalyzed by carboxypeptidase Y, this enzyme
does contain an alpha-carboxyl methyl esterase activity (41) which
may be active on the longer peptides found at intermediate
stages of proteolytic digestion and may result in the hydrolysis
of any L-isospartyl alpha-methyl ester linkages. It is also possible
that we have not detected the expected proteolytic products of
alpha-methyl esters because the proteolytic enzyme does not
cleave peptide bonds involving the beta-carboxyl group of iso-
erized aspartyl residues. Evidence that this is the case for carboxypeptidase Y has been presented under "Results" (cf.
Table II). Thus, we cannot presently determine the fraction
of D-aspartyl and L-isoaspartyl protein methyl esters which exist
in the total protein methyl esters of erythrocytes (2, 3)
or other cells.

-Isospartyl Hexapeptide As a Chemically Defined Methyl
Acceptor for Eucaryotic Protein Carboxyl Methyltransferases—
Up to now, all of the methyl-accepting substrates for the
carboxyl methyltransferase have been chemically undefined
subpopulations of peptides and proteins. However, with the
work presented here, as well as with that of Aswad (16),
highly homogeneous peptides are now described which are capable
of accepting close to one methyl group/polypeptide at the alpha-
carboxyl group of an isomerized L-aspartyl residue. The affinity
of these peptides for the methyltransferase (K_m = 1-6 M) is higher than that reported previously for other peptide and
protein substrates (63-67). It is significant that the maximal
velocity of the eucaryotic protein carboxyl methyltransferase
does not vary much between different protein and peptide
substrates (4, 64, 66), deamidated adrenocorticotropic hor-
mone (16), or the L-isospartyl hexapeptide described here.
These results suggest that the rate-limiting step of the enzy-

9 S. Clarke, unpublished observations.
10 C. M. O'Connor, D. W. Aswad, and S. Clarke, unpublished observations.

FIG. 8. Similar conformation of the beta-carboxyl group in a
D-aspartyl residue and the alpha-carboxyl group in a L-isospartyl
residue relative to the peptide backbone.
Protein Carboxyl Methylation at L-Isoaspartyl Residues

Implications for the Physiological Function of Protein Carboxyl Methyltransferases—Because of their chemical lability (43), eucaryotic protein methyl esters may not require an enzymatic activity for their hydrolysis in the cell. Their exceptional susceptibility to hydrolytic cleavage under physiological conditions can be understood if the hydrolysis involves a reaction such as an initial intramolecular attack of the carboxyl peptide nitrogen on the ester carbonyl group. Such a reaction will release methanol and form a hydrolytically labile succinimide ring. It should be noted that the same succinimide would be formed from either an aspartic acid β-methyl ester residue or an isoaspartic acid α-methyl ester residue. Nonenzymatic hydrolysis of the L-succinimide would produce both the L-normal aspartyl and the L-isoaspartyl residue (cf. Fig. 1). Repeated enzymatic methylation/nonenzymatic hydrolysis reactions would then be expected to result in a quantitative conversion of L-isoaspartyl residues to L-aspartic acid residues. If the L-isoaspartyl residue was originally formed by deamination of an L-asparaginyl residue, then a “repair” of the transpeptidation step, if not the deamidation step, is possible. It is also possible to invoke a scheme of racemization repair of D-aspartyl residues by a similar mechanism of enzymatic methylation and spontaneous demethylation if the succinimide intermediate is indeed racemization prone (cf. Ref. 10). Another possible intermediate in the nonenzymatic hydrolysis of protein methyl esters is an anhydride formed by the intramolecular attack of the carboxyl peptide oxygen on the ester carbonyl group. This intermediate may be subject to racemization at the α-carbon which could result in racemization repair of D-aspartyl residues by methylation/demethylation cycles. On the other hand, this intermediate could not be involved in the conversion of a methylated isoaspartyl residue to a normal aspartyl residue by a nonenzymatic process. At present, the contribution of each of these hydrolysis pathways to the demethylation of protein methyl esters in eucaryotic cells is not known.

Although we have been unable to identify an active esterase activity which catalyzes the hydrolysis of protein methyl esters in erythrocytes and others have detected only small amounts of activity in brain cytosol or other tissue extracts (5, 68), an activity has been detected in kidney which rapidly hydrolyzes gelatin [3H]methyl esters (58). The possibility of enzymatic demethylation is especially interesting to us because such enzymes could catalyze not only hydrolysis reactions but also transpeptidation, amidation, and epimerization reactions. In this way, L-isoaspartyl residues and D-aspartyl residues could be converted to L-aspartyl (or L-asparaginyl) residues in efficient repair reactions utilizing methylated intermediates (10). We are now in the process of identifying such enzymatic activities and repair pathways utilizing the synthetic peptides described here as substrates.

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