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Fragmentation of Isoaspartyl Peptides and Proteins by Carboxypeptidase Y: Release of Isoaspartyl Dipeptides as a Result of Internal and External Cleavage†

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ABSTRACT: Isoaspartate-containing versions of sea urchin sperm-activating peptide, δ sleep-inducing peptide, and lactate dehydrogenase (231–242) were cleaved at internal sites by carboxypeptidase Y. Cleavage occurred between the isoaspartate and the preceding amino acid, and it was accompanied by sequential digestion of amino acids from the two resulting carboxyl termini. Because the isoaspartyl bonds were not cleaved, isoaspartyl dipeptides were among the final products. The rate of release of isoaspartyl dipeptides was different for the three peptides, a 24-h digestion yielding 0.32 mol of isoaspartylglycine/mol of isoaspartyl sperm-activating peptide, 0.50 mol of isoaspartylalanine/mol of isoaspartyl δ sleep-inducing peptide, and 1.15 mol of isoaspartylserine/mol of isoaspartyl lactate dehydrogenase (231–242). The different rates could be explained by the slow cleavage of amino acids preceded by glycine. Isoaspartyl dipeptides were not detected in digests of the corresponding aspartate- or asparagine-containing forms of the peptides. Release of isoaspartyl dipeptides by carboxypeptidase Y was used to demonstrate the presence of isoaspartylglycine sequences in deamidated adrenocorticotropin (0.54 mol/mol), in a mixture of tryptic fragments of base-treated calmodulin (0.20 mol/mol), and in a mixture of tryptic fragments of base-treated triosephosphate isomerase (0.08 mol/mol). These results confirm earlier work suggesting that isoaspartylglycine formation is prevalent in proteins exposed to alkaline conditions. They also provide a methodology that should prove useful in the characterization of natural substrates for protein L-isoaspartyl methyltransferase.

Isoaspartyl (β-aspartyl) linkages form spontaneously in proteins and peptides following deamidation of certain asparagines or isomerization of certain aspartates (Gråf et al., 1971; Geiger & Clarke, 1987; Johnson et al., 1989b). When these atypical linkages, which are characterized by an extra carbon in the peptide backbone and a free α-carboxylic acid, are introduced into synthetic peptides, the peptides can be methylated by a protein carboxyl methyltransferase that shows no ability to methylate corresponding peptides containing

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L-aspartate, D-aspartate, or D-isoaspartate (Murray & Clarke, 1984; Johnson et al., 1987a; Galletti et al., 1989). The protein L-isoaspartyl methyltransferase also catalyzes the substoichiometric methylation of a wide range of proteins in vivo and in vitro, which implies the existence of isoaspartyl linkages in these proteins. It has been proposed that the methyltransferase might contribute to the repair or degradation of isoaspartate-containing polypeptides. The feasibility of the repair hypothesis has been demonstrated through in vitro studies showing that the methyltransferase can convert a majority of isoaspartyl linkages to normal α-aspartyl linkages (Johnson et al., 1987a; McFadden & Clarke, 1987; Galletti et al., 1988). This methylation-dependent conversion restores activity to calmodulin that has become inactivated as a result of in vitro aging (Johnson et al., 1987b).

A method for detecting isoaspartyl linkages independently of enzymatic methylation would be useful in testing hypotheses concerning the physiological function of the methyltransferase. In recent studies, isoaspartate has been detected by tritium labeling at internal α-carboxyl groups (Di Donato et al., 1986) and by a failure to sequence past sites originally occupied by asparagine or aspartate during automated Edman degradation (Gråf et al., 1971; Henderson et al., 1976; McDonald et al., 1983; Ekman et al., 1984; Kanaya & Uchida, 1986; Di Augustine et al., 1987; Johnson et al., 1989b). The tritium labeling method requires the use of high levels of ^3H_2O, and it would be subject to high background signals, especially if applied to complex mixtures in which some proteins might contain aspartate as the carboxyl-terminal amino acid. The Edman degradation strategy suffers because it requires the use of purified proteins, preferably of known sequence, and because failures in sequencing might have causes other than the presence of an isoaspartyl linkage.

In early studies investigating the sources of isoaspartyl peptides found in urine, proteins were digested with aggressive regimens of different proteases, followed by analysis of the digests for the presence of isoaspartyl di- and tripeptides (Haley et al., 1966; Pisano et al., 1966; Haley & Corcoran, 1967). Although these procedures yielded measurable amounts of isoaspartyl peptides, primarily isoaspartylglycine (Haley et al., 1966; Pisano et al., 1966; Haley & Corcoran, 1967), many of the isoaspartyl linkages appeared to have been formed in peptide fragments during the extensive incubations (Haley & Corcoran, 1967). It was therefore difficult to evaluate the original amount of isoaspartyl linkages in the proteins.

More recently, Murray and Clarke (1984) found that a synthetic version of adrenocorticotropin (22–27) containing isoaspartate at position 25 could be cleaved between positions 24 and 25 by carboxypeptidase Y (CPY),[1] an enzyme used for carboxyl-terminal sequencing of peptides and proteins (Jones et al., 1981). CPY apparently recognized the internal α-carboxylic acid of the isoaspartate and cleaved the bond between the isoaspartate and the preceding amino acid as if the isoaspartate with its β-carboxyl-linked peptide chain was a carboxyl-terminal amino acid (Murray & Clarke, 1984). CPY is active at relatively low pH (5.5–6.5) (Hayashi et al., 1975). Because low pH largely prevents the formation of isoaspartyl linkages, it seemed that proteolysis by CPY following a single endoprotease digestion might provide an excellent means by which one could estimate the original content of isoaspartyl linkages in proteins.

In this study, we show that CPY releases isoaspartyl dipeptides from three isoaspartyl oligopeptides of distinct sequence, but not from corresponding peptides containing aspartate or asparagine. We therefore used this method to demonstrate that an isoaspartylglycine sequence is present in deamidated adrenocorticotropin (ACTH), base-treated calmodulin, and base-treated triosephosphate isomerase, each of which is a substrate for the protein L-isoaspartyl methyltransferase (Aswad, 1984; Johnson et al., 1985, 1987b, 1989a).

Experimental Procedures

Proteins and Peptides. Calmodulin and the type I isoform of protein L-isoaspartyl methyltransferase were purified from bovine brain (Aswad & Deight, 1983). The native form of porcine ACTH (Sigma Chemical Co., St. Louis, MO) was isolated from minor contaminants by chromatography on CM-cellulose (Aswad, 1984). The deamidated form was generated by incubating a portion of this purified native ACTH for 6 h at 37 °C in 0.1 M NH_4OH, and it was isolated from the remaining native ACTH by chromatography on CM-cellulose (Aswad, 1984). The aspartyl and isoaspartyl versions of synthetic sea urchin sperm-activating peptide (speract) were generated by deamidation in 0.1 M NH_4OH at 37 °C for 6 h, and they were purified by reversed-phase high-performance liquid chromatography (HPLC) (Johnson et al. 1987a). The aspartyl and isoaspartyl versions of lactate dehydrogenase (231–242) [LDH(231–242)] were synthesized as described previously (Aswad et al., 1987). Isoaspartylserine was produced from α-aspartylserine by chemical methylation with 0.1 M HCl in methanol, followed by demethylation and hydrolysis of the resulting cyclic imide in 1 M ammonium acetate, pH 9.5 (McFadden & Clarke, 1986). CPY (catalog no. 238139) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Base Treatment of Proteins. Base treatment of calmodulin was in 0.1 M NH_4OH for 10 h at 37 °C followed by dialysis against 10 mM ammonium acetate, pH 7.4, and 0.1 mM EGTA. Base treatment of triosephosphate isomerase was in 0.2 M NH_4OH for 6 h at 37 °C followed by dialysis against 10 mM ammonium acetate, pH 7.4.

Methylation Reactions. Native and base-treated versions of calmodulin and triosephosphate isomerase were incubated at concentrations of 6–9 μM in methylation reactions containing 1 μM protein L-isoaspartyl methyltransferase and 200 μM *S*-adenosyl[*methyl*-^{3}H]-L-methionine (40–120 dpm/pmol). Reactions were carried out in a volume of 50 μL for 40 min at pH 6 and 30 °C and were terminated by adding 1 mL of 70 mg/mL trichloroacetic acid. Methyl groups in the precipitated protein were measured as described previously (Aswad & Deight, 1983).

Digestions of Synthetic Oligopeptides. CPY was dissolved in 20 mM pyridine acetate, pH 5.5, and 5% glycerol and dialyzed against the same buffer in order to remove free amino acids. Pronase was suspended in 20 mM sodium phosphate, pH 7.0, and dialyzed, after which glycerol was added to a final concentration of 5%. Small portions of each protease were stored at −70 °C and were thawed on the day of analysis.

Two hundred picomoles of synthetic peptide was suspended in 20 μL of either 0.1 mg/mL CPY or 0.06 mg/mL pronase. Digestions by CPY were carried out at 30 °C for 10 h, at which time an additional 10 μL of 0.1 mg/mL CPY was added, and digestion was continued for 14 h at 30 °C. Digestions by Pronase were carried out at 30 °C for 24 h. Each protease was also incubated in the absence of added peptide

[1] Abbreviations: ACTH, porcine adrenocorticotropin; CPY, carboxypeptidase Y; DSIP, δ sleep-inducing peptide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; OPA, *o*-phthaldialdehyde; speract, synthetic sea urchin sperm-activating peptide.

substrate in order to account for amino acids and isoaspartyl dipeptides produced upon autodigestion of the protease. All digestions were terminated by adding 20 μL of 20 mg/mL sodium dodecyl sulfate in 0.4 M sodium borate, pH 9.5. Stopped solutions were dried by centrifugation under vacuum and were then dissolved in 20 μM *S*-methylcysteine, which served as an internal standard for the ensuing amino acid analysis. Solutions were stored at −15 °C until analysis.

Measurement of amino acids and dipeptides released by the proteases was accomplished by using derivatization with *o*-phthaldialdehyde (OPA) followed by reversed-phase HPLC and fluorescence detection (Jones et al., 1981). Fluorescent derivatives were separated on a 3 mm × 10 cm Velosep C-18 column (Brownlee Labs, Santa Clara, CA) preceded by a 4.6 mm × 1 cm C-18 guard cartridge (Brownlee). The derivatives were detected with a Gilson Model 121 fluorometer using excitation at 305–395 nm and emission at 430–470 nm. The area under the peak of each amino acid and isoaspartyl dipeptide derivative was divided by the area of the *S*-methylcysteine internal standard in the same injection. The amount of each amino acid and isoaspartyl dipeptide released by the proteases was determined by comparing these values with those obtained for a mixture of standards.

Digestions of ACTH, Calmodulin, and Triosephosphate Isomerase. In triplicate, 42 μg of native or base-treated calmodulin was incubated for 22 h at room temperature with 1 μg of trypsin in 10 mM ammonium acetate, pH 7.4, and 0.1 mM EGTA. Triplicate incubations of 49 μg of native or base-treated triosephosphate isomerase were carried out similarly, except that the EGTA was omitted. The solutions were dried, resuspended in 100 μL of water, and dried again to remove the ammonium acetate. They were then resuspended in either 50 μL (calmodulin) or 100 μL (triosephosphate isomerase) of 0.1 mg/mL CPY in 10 mM pyridinium acetate, pH 5.5. Solutions were incubated for 24 h at 37 °C, at which time an amount of CPY equal to the first was added and incubation was continued for another 24 h at 37 °C. Native and deaminated ACTH were incubated at 100 μM concentration for 24 h at 37 °C in a total volume of 100 μL of 0.1 mg/mL CPY in 10 mM pyridinium acetate, pH 5.5. For each of the three digestion procedures, proteases were incubated in triplicate without protein substrate. Isoaspartyl dipeptide standards were also incubated throughout each digestion procedure in order to measure recoveries.

The CPY digests were then either subjected to chromatography on Dowex AG 1-X8 columns (calmodulin and ACTH) or injected directly for anion-exchange HPLC (triosephosphate isomerase). Dowex AG 1-X8 chromatography was carried out by using 1.5-mL columns equilibrated in 0.1 M acetic acid. After CPY digests were loaded, the columns were washed with 4 mL of 0.1 M acetic acid. Acidic amino acids and peptides were then eluted from the column with 5 M acetic acid. Fractions containing these acidic species were dried and resuspended in 100 μL of water. They were then injected for anion-exchange HPLC, which employed a 4 mm × 15 cm column of Aminex A-27 (Bio-Rad, Richmond, CA). Elution was achieved by using a linear gradient of acetic acid. When samples were injected for anion-exchange HPLC without prior Dowex AG 1-X8 chromatography, the Aminex A-27 column was preceded with a guard column containing the Dowex resin. Elution positions of aspartate, glutamate, isoaspartylglycine, isoaspartylserine, and isoaspartylalanine were determined from injections of standards. Fractions containing the isoaspartyl dipeptides of interest were collected, dried, resuspended in 20 μL of 0.4 M sodium borate, pH 9.5, and 20 mg/mL sodium dodecyl sulfate, and reacted with OPA as described for digests of synthetic peptides. Fluorescent derivatives were separated on a Beckman Ultrasphere ODS C-18 column.

Table I: Release of Isoaspartyl Dipeptides by CPY and Pronase

peptide	isoaspartyl dipeptide released[a] (mol/mol) CPY	Pronase
LDH(231–242)		
Asp-235	0.00	0.00
isoAsp-235	1.15	0.00
DSIP		
Asn-5	0.00	0.00
Asp-5	0.00	0.00
isoAsp-5	0.50	0.00
speract		
Asn-5	0.00	0.02
Asp-5	0.00	0.00
isoAsp-5	0.32	0.48

[a] Values are the means of duplicate injections.

Results and Discussion

Digestion of Asn-, Asp-, and IsoAsp-Containing Oligopeptides by CPY. Isoaspartyl versions of three synthetic oligopeptides were available for this experiment: lactate dehydrogenase (LDH) (231–242) (Lys-Gln-Val-Val-isoAsp-Ser-Ala-Tyr-Ile-Lys), δ sleep-inducing peptide (DSIP) (Trp-Ala-Gly-Gly-isoAsp-Ala-Ser-Gly-Glu), and speract (Gly-Phe-Asp-Leu-isoAsp-Gly-Gly-Gly-Val-Gly). The corresponding asparaginyl and aspartyl versions of DSIP and speract were also available, as was the aspartyl version of LDH(231–242). These peptides were incubated with CPY as described under Experimental Procedures, and the entire digestion mixtures were derivatized with OPA and injected for reversed-phase HPLC with fluorescence detection (see Figure 1 for a representative profile). The amount of isoaspartyl dipeptide was determined by comparing peak areas with appropriate standard dipeptides. Each of the peptides was also incubated with Pronase, a mixture of bacterial endo- and exopeptidase activities that was one of a series of proteases used in the past to measure the content of isoaspartyl linkages in proteins (Haley et al., 1966; Pisano et al., 1966; Haley & Corcoran, 1967).

Table I summarizes the yields of isoaspartyl dipeptides from each oligopeptide that was digested. CPY released isoaspartyl dipeptides from all of the isoaspartyl oligopeptides. However, the yield was different for the different peptides, ranging from about 1 mol of isoaspartylserine/mol of isoaspartyl LDH-(231–242) to about 0.3 mol of isoaspartylglycine/mol of isoaspartyl speract. No isoaspartyl dipeptides were detected in CPY digests of aspartyl or asparaginyl versions of the peptides, indicating that the digestion conditions did not generate isoaspartate through deamidation of asparagine or isomerization of aspartate.

Digestion of the peptides by Pronase gave different results (Table I). Although incubations of Pronase without peptide substrate contained amino acids at 10–30 μM, about 3 times the levels obtained for CPY, no isoaspartyl dipeptide was released from isoaspartyl versions of DSIP and LDH(231–242). Pronase did release more isoaspartylglycine from isoaspartyl speract than did CPY, but it also released a small amount of isoaspartylglycine from the asparaginyl version of the peptide, indicating deamidation and rearrangement of the peptide bond. Formation of isoaspartate during digestion by Pronase and not during digestion by CPY was probably a result of the higher pH that was used for the incubations with

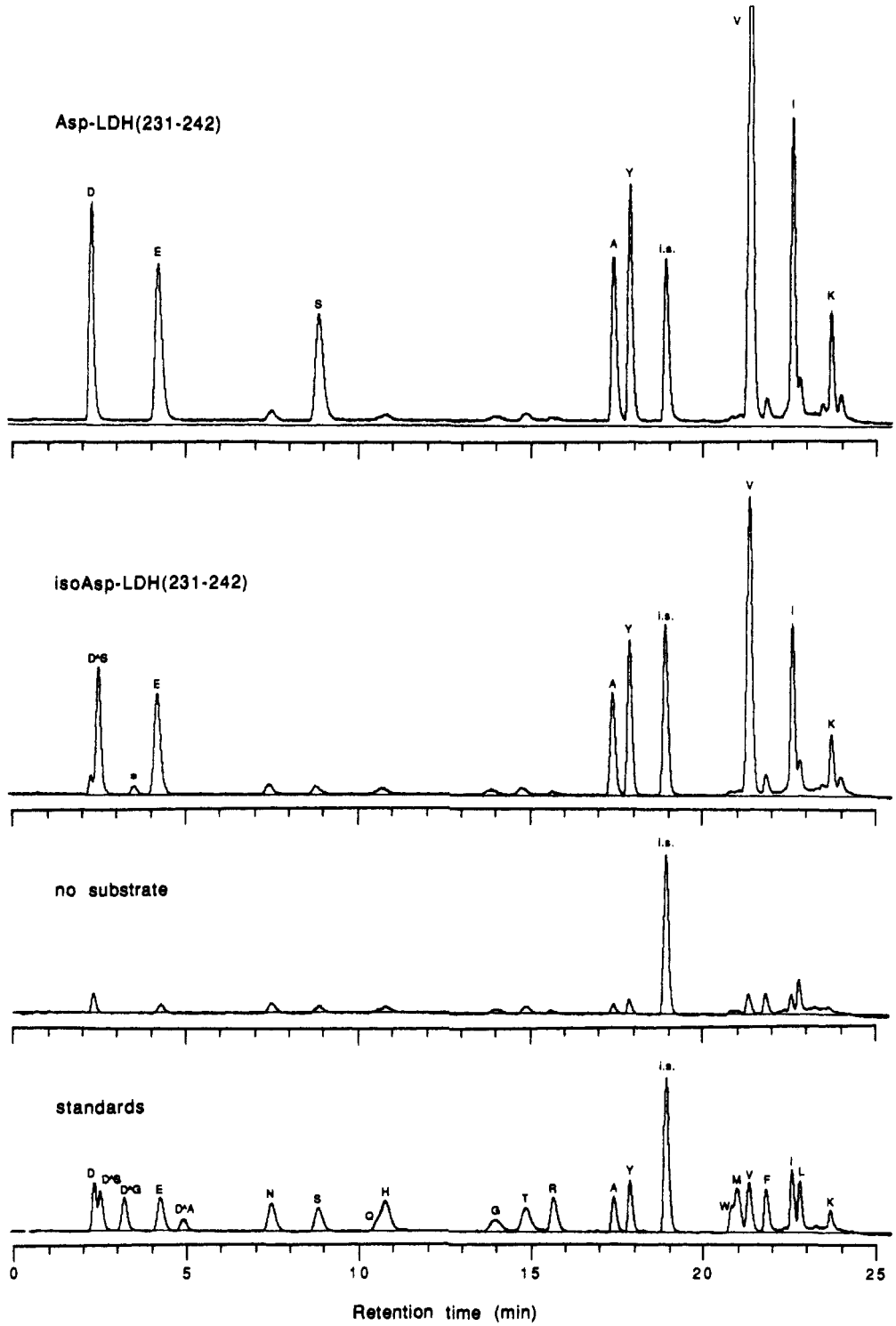

FIGURE 1: Fluorescence profiles for the determination of amino acids and isoaspartyl dipeptides released by CPY. Top panel: The aspartyl version of LDH(231–242) was incubated with CPY as described under Experimental Procedures. The entire digestion mixture was derivatized with OPA, and a portion of the derivatization reaction was subjected to reversed-phase HPLC with fluorescence detection. Second panel: A CPY digestion of the isoaspartyl version of LDH(231–242) was analyzed in the same manner as the aspartyl version. Third panel: CPY was incubated without added substrate in order to control for amino acids produced upon self-digestion of CPY. Bottom panel: A standard mixture of amino acids and isoaspartyl dipeptides was derivatized with OPA, and a portion containing 5 pmol of each derivative was subjected to HPLC. Each injection also contained 20 pmol of the internal standard (i.s.), *S*-methylcysteine. The symbol (^) represents an isoaspartyl peptide linkage. The asterisk (*) in the second panel denotes a derivative in the digest of isoaspartyl LDH(231–242) that was not present either in the digest of aspartyl LDH(231–242) or in the mixture of standards.

Pronase. Digestion by Pronase was carried out at pH 7 (the pH optima of the various peptidase activities in Pronase range from 6.5 to 8), whereas digestion by CPY was carried out at pH 5.5. The rate of deamidation of asparagine to isoaspartate is known to increase with increasing pH (Johnson et al., 1989b). The generation of isoaspartate from asparagine during digestion by Pronase and the inability of Pronase to release isoaspartyl dipeptides from two of the three isoaspartate-containing peptides tested indicate that CPY is a superior protease for detecting isoaspartate in proteins.

Reasons for the Incomplete Release of Isoaspartyl Dipeptides. We considered several possible explanations for the partial release of isoaspartyl dipeptides from isoaspartyl versions of DSIP and speract. The first possibility was that CPY

Table II: Digestion of LDH(231–242) Peptides, Lys-Gln-Val-Val-X-Ser-Ala-Tyr-Glu-Val-Ile-Lys, by Carboxypeptidase Y

	X = Asp		X = isoAsp	
	obsd[a]	expected[b]	obsd[a]	expected[b]
Ala	1.00 ± 0.05	1	0.96 ± 0.05	1
Asp	1.05 ± 0.05	1	−0.04 ± 0.00[c]	0
isoAsp-Ser	0.00 ± 0.00	0	1.15 ± 0.05	1
Gln	0.00 ± 0.00	0	0.00 ± 0.00	0
Glu	1.03 ± 0.03	1	1.04 ± 0.02	1
Ile	1.72 ± 0.09	1	0.99 ± 0.02	1
Lys	1.00[d]	1	1.00[d]	1
Ser	1.01 ± 0.07	1	0.04 ± 0.02	0
Tyr	0.98 ± 0.07	1	1.04 ± 0.00	1
Val	2.76 ± 0.18	3	2.52 ± 0.05	3

[a] Observed values represent the levels of each derivative in the peptide digest minus the levels in incubations of CPY without peptide substrate. They are reported as the means of duplicate determinations ± half the range. Only amino acids and isoaspartyl dipeptides possibly present in the analogues are shown. [b] Expected values assume that all carboxyl-terminal amino acids are released completely unless they are present in a dipeptide or in an isoaspartyl linkage. They also assume complete cleavage of the Val–isoAsp bond. [c] Negative values indicate that the amino acid was more abundant in incubations of CPY without substrate. [d] Values are normalized to 1.00 Lys, which is the carboxyl-terminal amino acid.

had cleaved the isoaspartyl linkage, releasing aspartate and its carboxyl-terminal neighbor. Another possibility was that CPY had failed to cleave the X–isoAsp bond completely. A third possibility was that digestion from the carboxyl terminus of the peptide had been incomplete, leaving a peptide of more than two amino acids that possessed isoaspartate at its amino terminus. Because the entire digestion mixtures were derivatized with OPA and injection for HPLC, it was possible to measure each amino acid released during the digestions and thus to determine the reasons for the varying yields of isoaspartyl dipeptides from the different isoaspartate-containing oligopeptides.

The yields of each amino acid released from the analogues of LDH(231–242) are shown in Table II, and they indicate that the digestion of these peptides was nearly complete. Aspartate and serine were released from the aspartyl version in quantities that were equal to that of lysine, the carboxyl-terminal amino acid of the peptide. Almost 3 mol of valine was released per mole of carboxyl-terminal lysine, but no glutamine was detected, indicating that digestion ceased after the formation of the amino-terminal dipeptide, Lys-Gln. This is consistent with previous observations that dipeptides are resistant to cleavage by CPY (Jones, 1986).

The results with the isoaspartyl version were similar, except that 1.15 mol of isoaspartylserine were released per mole of carboxyl-terminal lysine, and no aspartate or serine was detected. The fact that the release of isoaspartylserine was apparently stoichiometric and that 2.5 mol of valine was released per mole of carboxyl-terminal lysine indicates that cleavage of the Val–isoAsp bond was nearly quantitative. As indicated by an asterisk in Figure 1, there was a small fluorescent peak present in digests of the isoaspartyl version of LDH(231–242) that was not found in digests of the aspartyl version and that did not coelute with any of the stanard amino acid or isoaspartyl dipeptide derivatives. It is likely that this peak represents the tripeptide isoAsp-Ser-Ala. The additional alanine would increase the hydrophobicity of the derivative relative to isoaspartylserine and would therefore explain its increased retention. The absence of aspartate and serine from digests of the isoaspartyl version of LDH(231–242) indicates that the isoaspartyl bond was not cleaved by CPY. Control incubations of isoaspartylserine with CPY also showed no release of aspartate or serine.

Table III shows the yields of each amino acid and isoaspartyl dipeptide released during digestions of asparaginyl, aspartyl, and isoaspartyl versions of DSIP by CPY. Digestion of the asparaginyl and aspartyl versions appeared to be complete, because every amino acid present in the peptides was detected in the appropriate molar ratio to the carboxyl-terminal glutamate. There also appeared to be cleavage of the amino-terminal dipeptide, Trp-Ala, during digestions of both the asparaginyl and the aspartyl versions, as evidenced by the presence of tryptophan and by the fact that greater than 1 mol of alanine was released per mole of carboxyl-terminal glutamate.

A comparison of the results obtained for the isoaspartyl version of DSIP with those obtained for the asparaginyl and aspartyl versions indicated that the partial release of isoaspartylalanine from this peptide occurred because of incomplete cleavage of the Gly–isoAsp bond. As shown in Table III, serine was detected in digests of the isoaspartyl peptide at a level of nearly 1 mol/mol of carboxyl-terminal glutamate, implying quantitative release of residue 7. This indicates that CPY cleaved from the carboxyl terminus up to the isoaspartyl bond. No free alanine or aspartate was detected, indicating that no cleavage of the isoaspartyl linkage had occurred. Control incubations of isoaspartylalanine with CPY also showed no evidence of cleavage of the isoaspartyl bond. In order for complete release of serine-7 to have occurred, glycine-8 must have been released completely, as well. Because the total amount of glycine released was 1.34 mol/mol of the carboxyl-terminal glutamate, only 0.3–0.4 mol of glycine/mol of peptide must have been released from positions 3 and 4. The absence of tryptophan and alanine also suggests that CPY had little access to the bonds on the amino-terminal side of the isoaspartate. These results are all consistent with incomplete

Table III: Digestion of δ Sleep-Inducing Peptides, Trp-Ala-Gly-Gly-X-Ala-Ser-Gly-Glu, by Carboxypeptidase Y

	X = Asn		X = Asp		X = isoAsp	
	obsd[a]	expected[b]	obsd[a]	expected[b]	obsd[a]	expected[b]
Ala	1.39 ± 0.01	1	1.45 ± 0.02	1	0.07 ± 0.00	0
Asn	0.98 ± 0.03	1	−0.04 ± 0.06[c]	0	−0.02 ± 0.00[c]	0
Asp	0.00 ± 0.01	0	1.02 ± 0.05	1	0.00 ± 0.01	0
isoAsp-Ala	0.00 ± 0.00	0	0.00 ± 0.00	0	0.50 ± 0.00	1
Glu	1.00[d]	1	1.00[d]	1	1.00[d]	1
Gly	3.23 ± 0.08	3	5.37 ± 2.28	3	1.34 ± 0.08	3
Trp	0.66 ± 0.01	0	0.72 ± 0.01	0	0.06 ± 0.00	0
Ser	0.98 ± 0.01	1	0.99 ± 0.01	1	0.93 ± 0.02	1

[a] Observed values represent the levels of each derivative in the peptide digest minus the levels in incubations of CPY without peptide substrate. They are reported as the means of duplicate determinations ± half the range. Only amino acids and isoaspartyl dipeptides possibly present in the analogues are shown. [b] Expected values assume that all carboxyl-terminal amino acids are released completely unless they are present in a dipeptide or in an isoaspartyl linkage. They also assume complete cleavage of the Gly–isoAsp bond. [c] Negative values indicate that the amino acid was more abundant in incubations of CPY without substrate. [d] Values are normalized to 1.00 Glu, which is the carboxyl-terminal amino acid.

Table IV: Digestion of Sea Urchin Sperm-Activating Peptides, Gly-Phe-Asp-Leu-X-Gly-Gly-Gly-Val-Gly, by Carboxypeptidase Y

	X = Asn		X = Asp		X = isoAsp	
	obsd[a]	expected[b]	obsd[a]	expected[b]	obsd[a]	expected[b]
Asn	0.52 ± 0.05	1	−0.01 ± 0.00[c]	0	−0.04 ± 0.00[c]	0
Asp	0.56 ± 0.06	1	0.44 ± 0.04	2	0.82 ± 0.03	1
isoAsp-Gly	0.00 ± 0.00	0	0.00 ± 0.00	0	0.32 ± 0.02	1
Gly	2.76 ± 0.28	4	1.68 ± 0.10	4	1.74 ± 1.18	3
Leu	0.70 ± 0.08	1	0.30 ± 0.05	1	0.82 ± 0.03	1
Phe	0.04 ± 0.01	0	0.02 ± 0.01	0	0.16 ± 0.02	0
Val	0.88 ± 0.08	1	0.94 ± 0.06	1	0.31 ± 0.02	1

[a] Observed values represent the levels of each derivative in the peptide digest minus the levels in incubations of CPY without peptide substrate. They are reported as the means of duplicate determinations ± half the range and are expressed as fractions of the quantity of speract originally present in the digestion. Because the carboxyl-terminal amino acid, glycine, is present in multiple positions, and because digestion from the carboxyl terminus was not complete for the isoaspartyl form, no normalization to any individual amino acid was attempted. Only amino acids and isoaspartyl dipeptides possibly present in the analogues are shown. [b] Expected values assume that all carboxyl-terminal amino acids are released completely unless they are present in a dipeptide or in an isoaspartyl linkage. They also assume complete cleavage of the Leu–isoAsp bond. [c] Negative values indicate that the amino acid was more abundant in incubations of CPY without substrate.

cleavage of the Gly–isoAsp bond. The resistance of this bond to cleavage by CPY agrees with previous observations that glycine slows cleavage of a carboxyl-terminal amino acid when the glycine is present as the penultimate residue (Hayashi et al., 1975).

As shown in Table IV, asparaginyl and aspartyl versions of speract were more resistant to cleavage by CPY than were the corresponding forms of LDH(231–242) and DSIP. CPY released only 50–70% of the amino acids at positions 3–5 of the asparaginyl version, and the amount of glycine released was also very low. The extent of cleavage of the aspartyl version of speract was even lower than that of the asparaginyl form. The poor digestion of these peptides was probably caused by the slowing effect of glycine (Hayashi et al., 1975), which is very abundant in the carboxyl-terminal half of the peptides.

Release of amino acids from the carboxyl-terminal half of isoaspartyl speract was extremely poor relative to the aspartyl and asparaginyl forms, implying that isoaspartate can affect the metabolism of peptides at locations several amino acids away from the isopeptide bond. Only 0.3 mol of valine-9 was released per mole of peptide in the digestion mixture. However, there was good release of leucine-4 and aspartate-3 from isoaspartyl speract, indicating that CPY did cleave the Leu–isoAsp bond between residues 4 and 5. The presence of phenylalanine in the digest also suggests that CPY had access to the amino-terminal amino acids and that it was capable of cleaving partially the amino-terminal dipeptide, Gly-Phe. This result implies that the low level of isoaspartylglycine in the digest (0.3 mol/mol of peptide) was caused by incomplete digestion from the original carboxyl terminus of the peptide.

Complete cleavage of the Leu–isoAsp bond and partial cleavage from the carboxyl terminus should produce one or more peptides possessing an isoaspartate in the amino-terminal position. In contrast to most peptides (Taylor & Tappel, 1973; Schiltz et al., 1977), those containing amino-terminal isoaspartate give good fluorescent yields when derivatized with OPA (D. W. Aswad, unpublished results), so that peaks of fluorescence representing these products might be detected. In fact, we did observe a single large fluorescent peak eluting at 9.7 min in the digests of isoaspartyl speract that was not present either in digests of the aspartyl or asparaginyl versions or in the amino acid and isoaspartyl peptide standards.

Release of Isoaspartylglycine from Deamidated Proteins. The ability of CPY to release isoaspartyl dipeptides from each of the three isoaspartyl peptides, but not from asparaginyl or normal aspartyl forms, indicated that it might be suitable for analyzing isoaspartyl linkages in proteins. To test this, we chose to investigate the presence of isoaspartylglycine in deamidated porcine ACTH (39 residues), and in tryptic fragments of base-treated bovine brain calmodulin (148 residues) and base-treated rabbit muscle triosephosphate isomerase (248 residues). For these experiments, digestion by CPY was followed by partial purification of isoaspartyl dipeptides by anion-exchange chromatography (see Experimental Procedures) in order to reduce the total amount of amino acid reacted with OPA and to prevent large peaks of aspartate and glutamate from obscuring the isoaspartyl dipeptide derivatives on reversed-phase HPLC.

Table V: Isoaspartylglycine in Digests of Deamidated Proteins

protein	isoAsp-Gly (mol/mol)[b]	methylation by PIMT[a] (mol/mol)[b]
ACTH[c]		
native	0.01 ± 0.00	ND[f]
deamidated	0.54 ± 0.06	ND
calmodulin[d]		
native	0.02 ± 0.00	0.02 ± 0.00
base treated	0.20 ± 0.03	0.42 ± 0.04
triosephosphate isomerase[e]		
native	0.00 ± 0.00	0.00 ± 0.00
base treated	0.08 ± 0.02	0.22 ± 0.02

[a] PIMT, protein L-isoaspartyl methyltransferase. [b] Values are expressed as the mean ±SD of triplicate determinations. [c] IsoAsp-Gly values were corrected for a measured recovery of 40%. [d] IsoAsp-Gly values were corrected for a measured recovery of 32%. [e] IsoAsp-Gly values were corrected for a measured recovery of 60%. [f] ND, not determined.

Our first test of this strategy used ACTH, which is known to become deamidated at asparagine-25 to form isoaspartate, as judged by a failure of Edman degradation at this position (Gräf et al., 1971; Ekman et al., 1984). The deamidated form is also a substrate for the protein L-isoaspartyl methyltransferase from bovine brain (Aswad, 1984). Asparagine-25 lies in an Asn-Gly sequence, so that isoaspartylglycine should be released from deamidated ACTH if digestion by CPY went to completion. As shown in Table V, 0.54 mol of isoaspartylglycine/mol polypeptide was detected in digests of deamidated ACTH, whereas only 0.01 mol/mol was detected in digests of native ACTH. Because deamidation of ACTH produces a 70/30 mixture of isoaspartyl and aspartyl forms (Aswad, 1984; Johnson & Aswad, 1985), the 0.54 mol/mol release of isoaspartylglycine represents a 77% recovery of the isoaspartyl linkages originally present in the polypeptide.

The CPY digestion procedure was then applied to native calmodulin and to calmodulin that had first been incubated for 10 h at 37 °C in 0.1 M NH_4OH, conditions that cause an increase in its ability to be methylated by the protein L-isoaspartyl methyltransferase from bovine brain (Johnson et al.,

1985, 1987b, 1989a). Because calmodulin is a much larger protein than ACTH, it was first digested with trypsin so that CPY might have better access to internal isoaspartyl linkages. As shown in Table V, 0.20 mol of isoaspartylglycine was detected per mole of base-treated calmodulin, whereas only 0.02 mol/mol was detected in digests of the untreated protein. The base-treated calmodulin used for the digestions accepted 0.42 mol of methyl groups/mol of protein from the L-isoaspartyl methyltransferase. Thus, the isoaspartylglycine released by CPY can account for at least 48% of the isoaspartyl methyl-accepting sites. Because the studies with synthetic isoaspartyl peptides showed that CPY does not release isoaspartylglycine quantitatively from all sequences, the 0.20 mol/mol value may underestimate the isoaspartylglycine content of the base-treated calmodulin.

The isoaspartylglycine in base-treated calmodulin could have arisen from any of the two Asn-Gly or six Asp-Gly sequences present in the protein (Watterson et al., 1984). All of these candidate sequences are located in calcium-binding domains (Watterson et al., 1984), which is consistent with the observation that calcium can prevent the isoaspartate formation that occurs at neutral pH (Johnson et al., 1989a). Upon incubation at alkaline or neutral pH, the increase in the ability of calmodulin to accept methyl groups from the L-isoaspartyl methyltransferase is proportional to the amount of ammonia produced (Johnson et al., 1989a), and the increase can be largely blocked by a prior chemical modification of asparagine and glutamine side chains (Johnson et al., 1985), both of which observations suggest that most of the isoaspartate is generated upon deamidation of asparagines. Recent work in this laboratory has shown that asparagine-97, one of the asparagines in an Asn-Gly sequence, is the major site of isoaspartate formation induced by aging calmodulin at pH 7.4 in the absence of calcium (S. M. Potter and D. W. Aswad, unpublished results).

The final protein that was analyzed for the presence of isoaspartylglycine was triosephosphate isomerase, which is known to become deamidated at two Asn-Gly sequences upon exposure to elevated pH (Yuan et al., 1981). The deamidation of triosephosphate isomerase is accompanied by an increase in its ability to accept methyl groups from the L-isoaspartyl methyltransferase (Johnson et al., 1989a). As shown in Table V, 0.08 mol of isoaspartylglycine was released per mole of base-treated protein upon digestion by trypsin and CPY. Digests of untreated triosephosphate isomerase contained no detectable isoaspartylglycine. The base-treated triosephosphate isomerase that was subjected to digestion could be methylated to a level of 0.22 mol/mol by the protein L-isoaspartyl methyltransferase from bovine brain. Therefore, the isoaspartylglycine detected in the digests can account for at least 36% of the isoaspartyl methylation sites.

Conclusions. These experiments have shown that CPY is capable of releasing isoaspartyl dipeptides from peptides and proteins and that digestion by CPY might therefore be useful for detecting isoaspartyl linkages in proteins. The release of isoaspartyl dipeptide is usually not quantitative, and the yield varies depending on the amino acid sequence, so that negative results must be treated with caution. Nevertheless, if proper controls are carried out, the amount of isoaspartyl dipeptide detected should serve as a reliable minimum estimate of the content of isoaspartate in a protein or a mixture of proteins. The results with isoaspartyl speract show that even when an isoaspartyl dipeptide is not released, it may be possible to deduce the location of isoaspartate in a peptide of known sequence on the basis of the amino acids released from the carboxyl terminus produced upon internal cleavage by CPY.

The fact that CPY is capable of cleaving between isoaspartate and the preceding amino acid must be considered when the protease is used for gaining sequence information about the carboxyl terminus of proteins and peptides. If a portion of the substrate molecules possesses an isoaspartyl linkage, amino acids may be released from the new carboxyl terminus produced upon cleavage at the internal site. Such a release might compromise the validity of the sequence information, especially if the original carboxyl-terminal amino acid is blocked or cleaved slowly by the protease.

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Fluorescence Quenching in Model Membranes: Phospholipid Acyl Chain Distributions around Small Fluorophores†

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ABSTRACT: Fluorescence quenching in lipid bilayers is treated by a new approach based on calculation of the probability distribution of quenching and nonquenching acyl chains around a fluorophore. The effect of acyl lattice site dependence (i.e., correlations of phospholipid sister chain occupancy of neighbor sites) was modeled by use of Monte Carlo simulations of acyl chain occupancy. This explicit accounting of site occupancy correlation was found to fit observed quenching behavior better than did a model wherein phospholipid quenchers are considered to be independent. A key aspect of this approach is to evaluate the rate for quenching in a bilayer composed of pure quenching lipid. In order to evaluate this quenching rate, and also to provide a strong test of the calculated probability distributions, we synthesized lipids with both acyl chains labeled with a quenching moiety (Br or nitroxide), as well as the more usual single-chain quenchers. The fluorescence of tryptophan octyl ester (TOE), and of the 1,6-diphenyl-1,3,5-hexatriene (DPH) derivatives trimethylammonium-DPH (TMA-DPH) and 1-lauroyl-2-(DPH-propionyl)phosphatidylcholine (DPH-PC), was examined. We obtained consistent results with all the fluorophores and quenchers indicating that up to 18 neighboring acyl sites can contribute to quenching, corresponding to two shells of acyl sites on a hexagonal lattice. Calculated *discrete* distributions of fluorescence intensities were converted into fluorescence lifetimes and compared with Gaussian and Lorentzian *continuous* lifetime distributions. The fluorescence quenching theory presented here may be used to explain quantitatively the heterogeneity of fluorophore environments in multicomponent membranes.

Fluorescence quenching has been a useful technique for investigating the structure and dynamics of the environment of membrane-bound fluorophores. Quenchers of various sizes, charges, and hydrophobic character have quantitatively different effects on fluorescent membrane components. For example, the "depth" at which a fluorophore resides in the membrane, relative to the aqueous interface, can be inferred from its accessibility to different quenchers (Chattopadhyay & London, 1987; Thulborn & Sawyer, 1978). By varying the location of a quencher in the hydrophobic region of the membrane, a low-resolution map of intrinsically fluorescent tryptophans of a membrane-embedded polypeptide can be established, if the primary sequence is known (Markello et al., 1985). Relative lipid-protein binding constants can be determined with phospholipid analogues of quenchers (Caffrey & Feigenson, 1981; East & Lee, 1982; London & Feigenson, 1981b). Quenching behavior can also be influenced by partitioning of a fluorophore between phases giving different quantum yield, as occurs with lateral separations of gel and fluid phases in binary mixtures induced by temperature or ion binding (Feigenson, 1983; Florine-Casteel & Feigenson, 1988; Huang et al., 1988; Lentz et al., 1976; London & Feigenson, 1981c; Sklar et al., 1979; Yguerabide & Foster, 1981). In the absence of lateral phase separations, for membrane-bound proteins with intrinsically fluorescent tryptophans, the location and degree of exposure of tryptophan residues relative to the quencher determines the observed fluorescent intensity. Quenching of intrinsic tryptophanyl fluorescence has been investigated with brominated lipids (Berkhout et al., 1987; East & Lee, 1982; Leto et al., 1980) and nitroxide-labeled lipids (Bieri & Wallach, 1975; Caffrey & Feigenson, 1981; Haigh et al., 1979; London & Feigenson, 1978).

Fluorescence quenching, except for resonance energy transfer, requires close approach of the fluorophore and quencher. Significant reductions of fluorescence intensity are usually interpreted according to theories of either dynamic or static quenching, or a combination of both (Lakowicz, 1983). These theories have been applied successfully to a wide variety of quenching studies in isotropic, low-viscosity solutions, including the determination of tryptophan side-chain exposure in soluble proteins (Eftink & Ghiron, 1976; Lehrer, 1971) and the quenching of small organic fluorophores in organic solvents (Atik & Singer, 1978; Green et al., 1973).

The proper treatment of membrane-bound fluorophores and quenchers requires additional considerations. The membrane is an anisotropic medium where lipid components are constrained to two dimensions and diffuse at much lower rates than in nonviscous solvents. The composition of a model membrane can be manipulated to consist *entirely* of a quenching phospholipid analogue, thereby achieving effective

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