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Articles

Modification of Synthetic Peptides Related to Lactate Dehydrogenase (231-242) by Protein Carboxyl Methyltransferase and Tyrosine Protein Kinase: Effects of Introducing an Isopeptide Bond between Aspartic Acid-235 and Serine-236†

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ABSTRACT: The possibility that isoaspartyl residues contribute to the substrate specificity of eucaryotic protein carboxyl methyltransferases and/or tyrosine protein kinases has been investigated with two synthetic oligopeptides, Lys-Gln-Val-Val-Asp/isoAsp-Ser-Ala-Tyr-Glu-Val-Ile-Lys, which correspond to amino acids 231-242 of lactate dehydrogenase. One version of the peptide contains the normal amino acid sequence of the chicken muscle M_4 isozyme. The other version contains an isoaspartyl residue in position 235 in place of the normal aspartyl residue; i.e., Asp-235 is linked to Ser-236 via its side-chain β-carboxyl group, rather than via the usual α-carboxyl linkage. The normal peptide corresponds to the sequence around Tyr-238 that is phosphorylated in Rous sarcoma virus infected chick embryo fibroblasts [Cooper, J. A., Esch, F. S., Taylor, S. S., & Hunter, T. (1984) *J. Biol. Chem. 259*, 7835]. Using protein carboxyl methyltransferase purified from bovine brain, we found that the normal peptide did not serve as a methyl-accepting substrate but that the isopeptide served as an excellent substrate, exhibiting a stoichiometry of one methyl group per peptide and a K_m of 0.54 μM. With tyrosine protein kinase partially purified from normal rat spleen, both peptides were found to serve as phosphate acceptors at Tyr-238, exhibiting K_m values of 4.7 and 8.9 mM for the normal and isopeptide versions, respectively. These results support the idea that protein carboxyl methyltransferase selectively methylates the α-carboxyl group of atypical isoaspartyl residues. In contrast, the presence of isoaspartate had a modest negative effect on substrate activity for a tyrosine protein kinase from rat spleen.

The eucaryotic protein carboxyl methyltransferases (PCMTs)[1] and tyrosine protein kinases share the feature that they tend to covalently modify proteins both in vitro and in vivo with a low stoichiometry, usually less than 0.05 mol of methyl or phosphate group per mole of substrate (Kim & Li, 1979; Kloog et al., 1980; Aswad & Deight, 1983; Clarke & O'Connor, 1983; Sefton et al., 1981; Richert et al., 1982; Cooper et al., 1984). Recent experiments with PCMT from bovine brain and human erythrocytes suggest that this enzyme selectively methylates atypical L-isoaspartyl residues and not normal L-aspartyl or L-glutamyl residues (Aswad, 1984; Murray & Clarke, 1984). The possibility that many proteins contain low levels of isoaspartate, resulting from deamidation of asparagine, isomerization of normal aspartyl residues, or errors in protein synthesis, could explain the low stoichiometry of modification exhibited by this enzyme. To date, the only peptide for which stoichiometric methylation has been convincingly demonstrated is an isoaspartate-containing form of ACTH (Aswad, 1984; Murray & Clarke, 1984). It is important to determine if the apparent specificity of PCMT for isoaspartyl residues is a general property of this enzyme and the extent to which the specificity depends on the amino acid sequence surrounding the isoaspartate.

Recent studies on the amino acid sequences surrounding phosphorylation sites recognized by several tyrosine-specific protein kinases indicate that these enzymes may recognize

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[1] Abbreviations: ACTH, adrenocorticotropin; AdoMet, *S*-adenosyl-L-methionine; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(4-biphenylyl)-2-[(propyloxy)carbonyl]; Bzl, benzyl; cHex, cyclohexyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; PCMT, protein carboxyl methyltransferase; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Xan, xanthenyl; Z, benzyloxycarbonyl; all amino acids are the L isomer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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acidic amino acids in substrate proteins as determinants of specificity (Casnellie et al., 1982; Hunter, 1982; Pike et al., 1982; Swarup et al., 1983; Wong & Goldberg, 1983; Casnellie & Krebs, 1984). These and other observations (see below) prompted us to investigate whether isoaspartate might also have an effect on the specificity of this class of enzyme. Cooper et al. (1984) have compared the autophosphorylation site sequences in six different retroviral oncogene kinases with the phosphorylation site sequences of two glycolytic enzymes (lactate dehydrogenase and enolase) which are phosphorylated on tyrosine residues in Rous sarcoma virus infected chick embryo fibroblasts. Seven of the eight phosphorylation sites contained the consensus sequence -Asp-X-Y-Tyr- (or -Asp-X-Tyr-) where X is limited to Gly, Ser, Thr, or Asn. These same four amino acids are known to promote isoaspartate formation during peptide synthesis when they are coupled to the α-carboxyl group of (β-*O*-benzyl)-Asp (Baba et al., 1973; Bodanszky & Kwei, 1978; Ondetti et al., 1978; Yang & Merrifield, 1976). Moreover, it has been found that these same four amino acids are among the most common constituents of isoAsp-X dipeptides recovered from human urine, indicating that these sequences favor the formation of isoaspartyl linkages in proteins in vivo (Dorer et al., 1966). The formation of isoAsp–X bonds in peptides can arise from an alkaline-catalyzed deamidation of Asn-X peptides (Gräf et al., 1971; Bornstein & Balian, 1977) or an acid-catalyzed isomerization of Asp-X peptides (Swallow & Abraham, 1958; Naughton et al., 1960). It is of interest to note that Cooper et al. (1984) were able to enhance the phosphorylation stoichiometry of several substrates, including LDH, by preincubating them at low pH. They suggested that the low stoichiometry of substrate phosphorylation might be due to a selectivity of the tyrosine kinase for a distinct, atypical subpopulation of substrate molecules. Given the considerations just described, it seemed possible that these kinases might be selective for tyrosines positioned two or three residues on the carboxyl-terminal side of an atypical isoaspartyl bond.

We have unambiguously synthesized two peptides, LDH-(231–242) and (isoAsp^{235})LDH(231–242), corresponding to a sequence in the M_4 isozyme of chicken muscle LDH (Torff et al., 1977). Synthesis of the isopeptide version was carried out under conditions that should be generally applicable for synthesis of other isoaspartyl peptides. These peptide substrates have allowed us to address two specific questions. First, is the apparent specificity of PCMT for isoaspartyl residues, originally discovered in studies on ACTH and a synthetic derivative, generally applicable to other peptide sequences? Second, does the presence of an appropriately placed isoaspartyl residue influence the selectivity of a cellular tyrosine kinase for its substrate and thus explain the generally poor stoichiometry of this reaction?

Experimental Procedures

Purification of PCMT and Tyrosine Protein Kinase Activities. PCMT I was purified to ca. 90% homogeneity from bovine brain as described previously (Aswad & Deight, 1983). The enzyme was stored frozen at −15 or −70 °C in the presence of 5% (w/v) glycerol. A particulate fraction of normal rat spleen was used as the source of tyrosine protein kinase activity. It was prepared from fresh spleen of a 389-g female white Wistar rat exactly as described by Swarup et al. (1983) and stored at −15 °C in the presence of 10% (w/v) glycerol. The properties of this kinase have been extensively characterized by these authors. Their chromatographic and kinetic data suggest that the spleen particulate fraction consists of a single tyrosine kinase enzyme; however, the possibility that this fraction contains two or more similar enzymes was not ruled out.

Methyltransferase protein concentration was determined by the method of Lowry et al. (1957) using bovine serum albumin as a standard. Spleen particulate fraction protein was determined by the method of Bradford (1976) using a γ-globulin standard.

Methylation Reactions. Methylation reactions were all carried out at 30 °C in the pH 6.0–6.2 phosphate/citrate/EGTA buffer of Kim et al. (1978). Reaction mixtures contained a final volume of 50 μL and were initiated by addition of [*methyl*-^{3}H]AdoMet in a volume of 10 μL. The concentrations of methyltransferase, peptide substrate, and AdoMet, and the times of incubation, were varied as described in each experiment. Reactions were stopped by adding 50 μL of a solution containing 0.45 M sodium borate (pH 10.2), 4% (w/v) sodium dodecyl sulfate, and 2% (v/v) methanol. Release of tritiated methanol was measured by modification of the MacFarlane (1984) diffusion assay as described previously (Johnson & Aswad, 1985).

HPLC Analysis of LDH Isopeptide Methylation. The LDH isopeptide (9 μM) was incubated at 37 °C in 50 mM K-HEPES (pH 7.4)/1 mM EGTA with 2.5 μM PCMT and 200 μM [*methyl*-^{3}H]AdoMet for the indicated times. Reactions were stopped by adding an equal volume of 0.1% (w/v) trifluoroacetic acid. Samples containing 225 pmol of peptide were then injected into a 4.6 mm × 10 cm RP-300 reversed-phase column fitted with a 3-cm guard cartridge of the same material (Brownlee Labs). The column had been equilibrated with solvent A [0.1% (w/v) trifluoroacetic acid in water]. Solvent B was water containing 0.1% (w/v) trifluoroacetic acid and 90% (v/v) acetonitrile. The following solvent flow profile was carried out at 1.0 mL/min: 10% B for 2 min, then a linear increase to 18% B over 1 min, and then hold at 18% B for 10 min. Peptide absorbance was monitored at 214 nm with a Kratos 757 detector. Fractions of 250 μL were collected and counted in 1 mL of ACS scintillation fluid (Amersham).

Phosphorylation Reactions. Phosphorylation reactions [modeled after those of Casnellie et al. (1982) and Swarup et al. (1983)] were all carried out at 30 °C in a 50-μL volume containing 50 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 10 μM sodium vanadate, 0.5% (v/v) Triton X-100, peptide (at the indicated concentrations), spleen particulate fraction (0.36–0.40 mg/mL protein), and [γ-^{32}P]ATP (concentration and specific activity as indicated in figure legends). Reactions were initiated by adding the [γ-^{32}P]ATP in a volume of 5 μL. Reactions were stopped by adding 150 μL of 3.3% (w/v) trichloroacetic acid and 10 μL of bovine serum albumin (20 mg/mL) as carrier. Incorporation of ^{32}P into the acid-soluble peptide was assessed by the phosphocellulose paper binding method (Glass et al., 1978; Swarup et al., 1983).

Analysis of Phosphoamino Acids. The LDH peptides were individually phosphorylated by rat spleen particulate kinase as described above with 600 μM peptide and 50 μM [γ-^{32}P]ATP (ca. 2000 cpm/pmol) for 10 min. After termination of the reaction with trichloroacetic acid, the supernatant, containing the peptide, was transferred to a clean 1.5-mL microfuge tube, reduced to dryness, resuspended in 0.5 mL of ether, and then reduced again to dryness. The residue was dissolved in 0.1% (w/v) trifluoroacetic acid, and unreacted ATP was separated from peptide material on a 3-cm RP-300 HPLC reversed-phase guard column (Brownlee Labs). Unreacted ATP was eluted from the column with 5% (v/v) acetonitrile containing 0.1% trifluoroacetic acid. The peptide was then eluted with an aqueous solution of 30% acetonitrile

containing 0.1% (w/v) trifluoroacetic acid.

The peptide fractions were reduced to dryness and then subjected to hydrolysis in 6 N HCl at 110 °C for 2 h. The hydrolysates were reduced to dryness, and each was dissolved in 10 μL of electrophoresis buffer (pyridine/acetic acid/water, 1:10:189, pH 3.5). Each peptide (2.5 μL) was spotted on 20-cm thin-layer cellulose sheets, alongside a standard mixture containing 2.5 nmol of phosphoserine, phosphothreonine, and phosphotyrosine. Electrophoresis was carried out at 1000 V for 40 min. The standards were localized with ninhydrin, and the [^{32}P]phosphoamino acids were localized by autoradiography on Kodak XAR film.

N^{α}-tert-Butyloxycarbonyl-L-aspartic Acid (α-O-Cyclohexyl). This protected amino acid was synthesized in two steps essentially as described by Tam et al. (1979) for the corresponding β-cHex ester except that 4-(dimethylamino)pyridine was not used as a catalyst. Boc-Asp(β-*O*-Bzl) (10 g, 31 mmol) was dissolved in 30 mL of CH_2Cl_2 containing cyclohexanol (9.7 mL, 93 mmol) and cooled to 0 °C. Then 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (6 g, 31 mmol) was added, and the mixture was stirred at 0 °C for 20 h. The mixture was rotoevaporated to an oil that was distributed between ethyl acetate and 1 N H_2SO_4. The aqueous acid was extracted once more with ethyl acetate. The pooled ethyl acetate extracts were washed twice with water, once with saturated NaCl, twice with 5% $NaHCO_3$, and again with water and saturated NaCl. The ethyl acetate was dried over anhydrous Na_2SO_4 and rotoevaporated to an oily residue of Boc-Asp(α-*O*-cHex)(β-*O*-Bzl): yield 12 g (29.6 mmol, 96%); TLC on silica gel G in chloroform/methanol/acetic acid (17:2:1), R_f 0.93, and in chloroform/methanol (10:1), R_f 0.67 (single spots). Detection was by ninhydrin spray after exposure of chromatograms to concentrated HCl vapors for 10 min. Boc-Asp(α-*O*-cHex)(β-*O*-Bzl) stained yellow. No ninhydrin reaction occurred on replicate chromatograms without acid treatment.

Boc-Asp(α-*O*-cHex)(β-*O*-Bzl) was converted to Boc-Asp(α-*O*-cHex) by catalytic hydrogenation. The above oil was dissolved in 90 mL of 95% ethanol, 5% Pd/$BaSO_4$ (10 g) was added, and H_2 was bubbled through the mixture for 2 h. The catalyst was removed by centrifugation, and the supernatant was rotoevaporated to an oily residue. The oil was distributed between ethyl acetate and ice-cold 20% citric acid. The aqueous acid was reextracted once with ethyl acetate, and the pooled ethyl acetate extracts were washed twice with water and once with saturated NaCl. The ethyl acetate was then washed twice with saturated NaCl in 5% $NaHCO_3$ to remove an uncharacterized contaminant (R_F 0.72 in chloroform/methanol/acetic acid) from Boc-Asp(α-*O*-cHex), although these extractions also resulted in appreciable loss of the desired product. The ethyl acetate was finally washed twice with saturated NaCl, dried, and rotoevaporated to an oil. After analysis, the product was dissolved in 20 mL of CH_2Cl_2 for coupling during peptide synthesis: yield 2.2 g (7 mmol, 24%); TLC on silica gel G in chloroform/methanol/acetic acid (17:2:1), R_f 0.84, and in chloroform/methanol (10:1), R_f 0.42 (single spots). The reaction product was chromatographed with standards of Boc-Asp, Boc-Asp(β-*O*-cHex), Boc-Asp(α-*O*-Bzl), and Boc-Asp(β-*O*-Bzl). The latter three standards had R_f values identical with that of Boc-Asp(α-*O*-cHex). After treatment with HCl vapor, Boc-Asp(α-*O*-cHex) and the Boc-Asp(α-*O*-Bzl) standard stained blue-purple, whereas β-protected Boc-Asp standards stained orange. No ninhydrin staining occurred without prior acid treatment of chromatograms.

A sample of Boc-Asp(α-*O*-cHex) product and standards of the corresponding β-cHex, α-Bzl, and β-Bzl esters were treated with 40% TFA in CH_2Cl_2 for 20 min at room temperature to remove the Boc group. Following several rotoevaporations to remove TFA, the residues were dissolved in 80% formic acid and subjected to thin-layer electrophoresis at pH 3.5 (pyridine/acetic acid/water, 1:10:289) on cellulose sheets at 1000 V for 40 min followed by ninhydrin staining. Asp(α-*O*-cHex) and Asp(α-*O*-Bzl) had R_f values of −2.32 (relative to Asp standard, R_f 1.00) and stained purple, while Asp(β-*O*-cHex) and Asp(β-*O*-Bzl) had R_f values of −1.04 and stained orange. Also, Boc-Asp(α-*O*-cHex) could be quantitatively converted to free Asp by treatment with anhydrous HF.

Peptide Synthesis and Purification. LDH(231–242) and (isoAsp^{235})LDH(231–242) were manually synthesized by solid-phase techniques using a double-coupling protocol (Glass, 1983). The peptide was started on a 1-mmol scale using 1% cross-linked chloromethylated resin that had been esterified (0.49 mmol/g) to Boc-Lys(2-chloro-Z). After synthesis through serine-236, the peptidyl resin was divided in half. Boc-Asp(β-*O*-cHex) and Boc-Asp(α-*O*-cHex) were used in completing the syntheses of LDH(231–242) and (isoAsp^{235})LDH(231–242), respectively. In addition, the following Boc-L-amino acids were used: Gln(Xan), Val, Ser(*O*-Bzl), Ala, Tyr(Cl_2-Bzl), Glu(*O*-Bzl), and Ile·$^1/_2H_2O$. Dicyclohexylcarbodiimide was the coupling reagent. The second coupling of Boc-Asp(α-*O*-cHex) in (isoAsp^{235})LDH(231–242) was conducted in the presence of an equimolar amount of hydroxybenzotriazole. The Boc group of Boc-Gln(Xan) was removed with 4 N HCl in dioxane.

Half of each peptidyl resin was cleaved with anhydrous HF/anisole (10:1) for 45 min at −20 °C. The remaining samples were cleaved by the low-HF/high-HF procedure of Tam et al. (1983). After removal of residual scavenger with ethyl acetate, peptide was extracted from the resins with 1 N acetic acid. Both crude peptides were purified by ion-exchange chromatography on SP-Sephadex (Kemp et al., 1976). Final purification of (isoAsp^{235})LDH(231–242) from two minor contaminants was achieved by partition chromatography (Yamashiro, 1964) using the buffer system described by Glass et al. (1985).

Concentrations of the peptides were determined by the absorbance at 274.5 nm using the extinction coefficient of 1413 M^{-1} cm^{-1} for tyrosine (Segel, 1968) and by quantitative amino acid analysis (Heinrikson & Meredith, 1984) of duplicate acid hydrolysates (5.7 N HCl, 110 °C, for 24, 48, and 72 h).

Results and Discussion

Characterization of the LDH Peptides. The sequences of the normal (eq 1) and isoAsp (eq 2) versions of LDH(231–242) are shown. The LDH(231–242) sequence was chosen

Lys-Gln-Val-Val-Asp-Ser-Ala-Tyr-Glu-Val-Ile-Lys (1)

Lys-Gln-Val-Val-isoAsp-Ser-Ala-Tyr-Glu-Val-Ile-Lys (2)

for synthesis because it corresponds to the site of tyrosine phosphorylation in LDH, it contains an aspartyl residue NH_2-terminal to that tyrosine, and suitable peptides of that size can serve as substrates for either tyrosine protein kinases or PCMTs. Lysyl residues at positions 231 and 242 in the sequence were included so that the peptides would have a 3+ net charge (prior to phosphorylation) at pH 2. Under these conditions, phosphopeptides would be quantitatively bound to phosphocellulose paper squares (Glass et al., 1978) so a rapid, convenient assay of phosphotransferase activity could be used for these substrates.

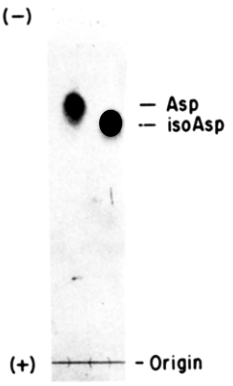

FIGURE 1: Thin-layer electrophoresis of LDH peptides. Approximately 8.5 nmol of each peptide was spotted at the origin and subjected to electrophoresis in pyridine/acetic acid/water (1:10:189, pH 3.5) for 90 min at 500 V. The Asp-235 form migrated 10.3 cm from the origin, while the isoAsp-235 form migrated 9.6 cm.

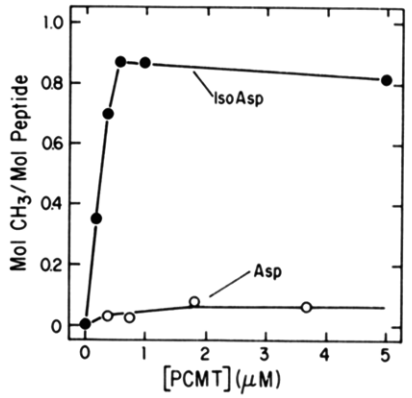

FIGURE 2: Methylation of LDH peptides. Methylation reactions were carried out for 30 min as described under Experimental Procedures. Peptides were present at 10 μM, and [*methyl*-^{3}H]AdoMet was present at 200 μM (92 dpm/pmol). The concentration of PCMT was varied as indicated by the abscissa to ensure that maximal methylation was reached.

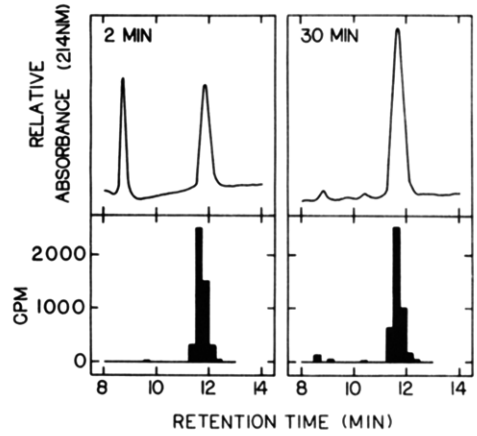

FIGURE 3: HPLC analysis of LDH peptide methylation. The LDH isopeptide was methylated by PCMT at 37 °C, pH 7.4, for the indicated times and then subjected to HPLC as described under Experimental Procedures. The pure, unincubated isopeptide yielded a single peak at 8.7 min (not shown). At 2 min, the relationship between cpm and peptide absorbance indicated that virtually all of the material eluting at 11.6 min was methylated peptide. At 30 min, however, only about half of the absorbing material was methylated. Subsequent experiments have demonstrated that the unmethylated fraction of this 11.6-min peak is the cyclic imide (aspartimide) form of the peptide which results from rapid nonenzymatic demethylation of the isoaspartate methyl ester. A similar finding was previously demonstrated for methylation of deamidated ACTH(1–39) (Johnson & Aswad, 1985).

As discussed above, a benzyl-protected Asp-Ser sequence in the desired LDH peptides is susceptible to cyclic imide formation during peptide synthesis (base-catalyzed) and subsequent HF cleavage and deprotection (acid-catalyzed). Any aspartimide-containing peptide can subsequently reopen to a combination of aspartyl and (predominantly) isoaspartyl products that would require complete resolution before the desired experiments could be conducted. Unambiguous synthesis of the two peptides in which this side reaction is minimized or prevented could be approached by (i) use of Bpoc-protected amino acids in combination with the acid-labile *tert*-butyl ester protection of carboxyl side chains on the *p*-alkoxybenzyl ester resin (Wang et al., 1974), (ii) use of phenacyl ester protection for Boc-Asp with removal of this carboxyl side chain protecting group prior to HF cleavage (Yang & Merrifield, 1976), or (iii) use of either cyclopentyl (Blake, 1979) or cyclohexyl protecting groups (Tam et al., 1979) for Boc-Asp, which were designed to reduce aspartimide formation. We chose to use Boc-Asp(β-*O*-cHex) and Boc-Asp(α-*O*-cHex) for the synthesis of LDH(231–242) and (isoAsp^{235})LDH(231–242), respectively. The β-protected Boc-amino acid was commercially available, and the α-protected one was synthesized as described under Experimental Procedures. This synthetic strategy was combined with HF cleavage procedures that would minimize aspartimide formation, either cleavage at −20 °C (Tam et al., 1979) or the recently introduced low-HF/high-HF cleavage procedure (Tam et al., 1983). In addition to synthesis of isoaspartyl-containing peptides as substrates of PCMTs, Boc-Asp(α-*O*-cHex) could be used for the synthesis of COOH-terminal asparagine peptides on benzhydrylamine resins. This approach would be especially useful for longer peptides, since both the cyclohexyl ester bond and the benzhydrylamine resin amide bond are appreciably more stable to 50% TFA/CH_2Cl_2 than are benzyl ester protection and the classical benzyl ester resin bond (Tam et al., 1979; Pietta et al., 1973).

The amino acid composition of LDH(231–242) was Asp (1.00), Glx (1.91), Ser (0.96), Ala (1.08), Tyr (0.96), Val (2.93), Ile (1.18), and Lys (2.01); that of its isoAsp-containing analogue was Asp (1.05), Glx (1.96), Ser (0.97), Ala (1.07), Tyr (0.92), Val (2.95), Ile (1.15), and Lys (1.95). Yields of the purified portions of LDH(231–242) and (isoAsp^{235})-LDH(231–242) from the −20 °C HF cleavage were 16% and 25%, respectively (based on starting resin). Each peptide was pure as assessed by ninhydrin staining after TLC on cellulose sheets in solvent A (Glass & Smith, 1983) and by HPLC as described under Experimental Procedures. Minor peaks obtained during peptide purification were not fully characterized.

Figure 1 shows the results of subjecting the two synthetic peptides to thin-layer electrophoresis at pH 3.5. Both peptides exhibited a single ninhydrin-positive spot running toward the cathode. As expected, the isopeptide form exhibited a slightly slower migration, due to the more acidic nature of the α-carboxyl side chain of the isoAsp residue.

Carboxyl Methylation of the LDH Peptides. To assess the methyl-accepting capacities of the normal and isopeptide versions of LDH(231–242), each peptide was incubated at a concentration of 10 μM with 200 μM [*methyl*-^{3}H]AdoMet and varying amounts of PCMT in a standard 40-min assay. As shown in Figure 2, the isopeptide could be methylated to a maximum stoichiometry of 87 mol %, whereas the normal peptide exhibited a barely detectable methylation over the entire range of PCMT levels.

Figure 3 shows an HPLC analysis of the isopeptide after 2 or 30 min of methylation under conditions similar to those

used in Figure 2. In this HPLC system, the pure isopeptide runs as a single peak at 8.7 min. After 2 min of methylation, a new peak appears at 11.6 min. The distribution of radiolabel indicates that only the latter peak is methylated. By 30 min, greater than 95% of the isopeptide is gone, indicating that the modification is stoichiometric. The value of 87 mol % methylation obtained in Figure 2 probably underestimates slightly the true extent of methylation due to limits on the accuracy of the peptide concentration and the specific activity of the [^{3}H]AdoMet and to variations in recovery of [^{3}H]methanol in the diffusion assay.

To compare the specificity of PCMT for the LDH isopeptide with its specificity for other isopeptides, methylation was studied under initial rate conditions over isopeptide concentrations ranging from 0.06 to 2.0 μM. The data yielded a linear Lineweaver–Burk plot with an apparent K_m of 0.54 μM and a V_{max} of 22 nmol min^{-1} mg^{-1} (data not shown). These are the most favorable kinetic constants found to date for any substrate for a eucaryotic PCMT.

The LDH isopeptide now provides a second example where stoichiometric carboxyl methylation of a peptide of defined sequence by a eucaryotic PCMT has been convincingly demonstrated. Previous studies with deamidated ACTH or synthetic peptides corresponding to deamidated ACTH(22–27) revealed that both the bovine brain and human red cell forms of PCMT selectively methylate an isoaspartyl residue, but not a normal aspartyl residue, in position 25 of this hormone (Aswad, 1984; Murray & Clarke, 1984). The K_m of bovine brain PCMT for the isoaspartate-containing form of deamidated ACTH(1–39) is approximately 1.3 μM. The fact that two unrelated isoaspartyl peptides (LDH and ACTH) both exhibit K_m values in the low micromolar range strongly suggests that the isoaspartyl residue itself is the major factor in determining recognition by the active site of PCMT. Ongoing experiments with other unrelated peptide sequences support this conclusion.[2]

A specificity of PCMT for isoaspartate-containing peptides has important implications for the function of this enzyme. PCMT-catalyzed methylation in eucaryotes was originally believed to be involved in reversible regulation of protein function [for reviews, see Gagnon & Heisler (1979), Paik & Kim (1980), and O'Dea et al. (1981)], as it is in chemotactic bacteria (Springer et al., 1979; Koshland, 1981). Such a regulatory role, coupled with the unusual specificity of PCMT for isoaspartyl residues, implies that isoaspartate might be a normal component of certain cellular proteins, i.e., those which are the targets of regulation. More recently, it has been proposed that PCMT may be involved in the repair or degradation of isoaspartate-containing proteins (Aswad, 1984; Murray & Clarke, 1984, 1986; Johnson & Aswad, 1985; Johnson et al., 1985). In this latter theory, isoaspartate is viewed as an abnormal and deleterious component of proteins arising from spontaneous deamidation of labile asparagine residues, isomerization of aspartyl residues, or errors in protein synthesis. A strong dependence of PCMT specificity for unique sequences surrounding the isoaspartate would seem to favor a regulatory role for this enzyme. The cyclic nucleotide dependent protein kinases, for example, show a strong dependence on the sequences surrounding the phosphorylation sites in their respective oligopeptide substrates [reviewed by Glass & Krebs (1980)]. Conversely, a lack of sequence dependence would appear to favor the abnormal protein recognition hypothesis since it is the presence of the abnormal aspartate linkage, rather than other determinants in the protein, that is of primary importance. More data on the specificity of PCMT for isoaspartate-containing oligopeptides with a variety of distinct sequences should help to determine the validity of this conclusion.

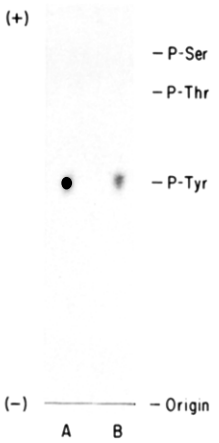

FIGURE 4: Identification by thin-layer electrophoresis of phosphotyrosine in LDH peptides phosphorylated by the rat spleen particulate fraction. Hydrolysates of the phosphorylated normal and isoaspartyl-LDH(231–242) peptides were spotted in lanes A and B, respectively. An autoradiogram of the electrophoresis plate is shown, with positions of the phosphoamino acid standards indicated. Other details of analysis are described under Experimental Procedures.

Tyrosine Phosphorylation of the LDH Peptides. As demonstrated by Swarup et al. (1983), rat spleen is a rich source of tyrosine protein kinase activity in normal tissue and has proven useful in delineating the substrate specificity of this class of enzyme. Using this preparation, we found that both LDH peptides, tested in phosphorylation reactions containing 332 μM peptide and 600 μM ATP, were roughly comparable as phosphate acceptors. Using a 10-min reaction at 30 °C under these conditions, we obtained phosphorylation rates of 840 and 703 pmol min^{-1} (mg of spleen protein)$^{-1}$ for the normal and isopeptide forms of LDH(231–242), respectively. These rates are corrected for an endogenous rate (measured as the phosphate incorporation in the absence of added peptide) of 10.9 pmol min^{-1} mg^{-1}. Our peptide phosphorylation rates are very similar to the rate of the best peptide, Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly, tested by Swarup et al. (1983), which exhibited a phosphorylation of 668 pmol min^{-1} mg^{-1} at 1.0 mM peptide and 60 μM ATP.

Since our LDH peptides contain serine, in addition to tyrosine, it was important to establish the site of phosphorylation. Both LDH peptides were phosphorylated by the rat spleen particulate enzyme, separated from unreacted ATP by reversed-phase HPLC, and then partially hydrolyzed. The hydrolysates were subjected to thin-layer electrophoresis along with standards of phosphoserine, phosphothreonine, and phosphotyrosine. Autoradiography of the electrophoresis sheet (Figure 4) revealed that phosphate incorporation occurred almost exclusively into tyrosine with both peptides.

To determine whether the isopeptide bond has any quantitative effect on the specificity of the spleen kinase, we carried out phosphorylation assays under initial rate conditions over peptide concentrations ranging from 0.5 to 2.0 mM. The results are shown in Figure 5. Both peptides yielded a V_{max} of approximately 4.4 nmol min^{-1} mg^{-1}. The apparent K_m values for the normal and isopeptide forms were 4.7 and 8.9 mM, respectively. From these data, it appears that the isopeptide bond has a mild negative effect on the tyrosine kinase activity.

[2] B. A. Johnson and D. W. Aswad, unpublished observation; S. Clarke, personal communication.

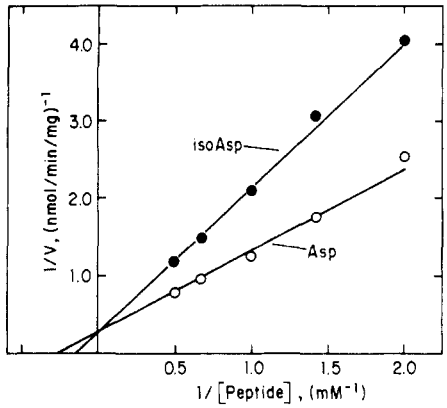

FIGURE 5: Lineweaver–Burk plot for phosphorylation of the LDH peptides by rat spleen tyrosine protein kinase. Phosphorylation reactions were carried out for 10 min in the presence of 50 μM [γ-^{32}P]ATP (930 cpm/pmol).

As discussed in the introduction, there were several reasons to investigate the possibility that isoaspartyl residues might have a significant effect on the specificity of tyrosine protein kinase. Had the LDH isopeptide exhibited a substantially lower K_m than the normal peptide, this could have provided a possible explanation for the low stoichiometries of tyrosine phosphorylation which are typically observed (at least for some substrates) both in vivo and in vitro. Such a result would imply that phosphorylation occurred selectively on a minor fraction of the LDH molecules that contained isoaspartate in position 235.

Our results indicate this is not the case and that other factors must be responsible for the low stoichiometries of phosphorylation catalyzed by tyrosine protein kinases. As suggested by Cooper et al. (1984), phosphorylation may be limited to a denatured, or otherwise modified, subpopulation of substrate molecules which fortuitously assume a conformation that enhances the susceptibility of the appropriate tyrosine to phosphorylation. This idea is supported by studies of Richert et al. (1982), who found evidence that the *src* kinase selectively phosphorylates an atypical subpopulation of casein molecules. Finding the natural substrates for both normal cellular and oncogene-derived tyrosine protein kinases remains a challenging problem if one is to understand the role these enzymes play in normal development and in oncogenic transformation.

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Limited Proteolysis of Type I Collagen at Hyperreactive Sites by Class I and II *Clostridium histolyticum* Collagenases: Complementary Digestion Patterns†

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ABSTRACT: The initial proteolytic events in the hydrolysis of rat tendon type I collagen by the class I and II collagenases from *Clostridium histolyticum* have been investigated at 15 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis has been used to detect the initial cleavage fragments of both the α1(I) and α2 chains, which migrate at different rates in the buffer system employed. Experiments with the class I collagenases indicate that the first cleavage occurs across all three chains of the triple helix close to the C-terminus to produce fragments whose α chains have molecular weights of approximately 88 000. The second cleavage occurs near the N-terminus to reduce the molecular weight of the α chains to 80 000. Initial proteolysis by the class II collagenases occurs across all three chains at a site in the interior of the collagen triple helix to give N- and C-terminal fragments with α-chain molecular weights of 35 000 and 62 000, respectively. The C-terminal fragment is subsequently cleaved to give fragments with α-chain molecular weights of 59 000. These results indicate that type I collagen is degraded at several hyperreactive sites by these enzymes. Thus, initial proteolysis by these bacterial collagenases occurs at specific sites, much like the mammalian collagenases. These results with the individual clostridial collagenases provide an explanation for earlier data which indicated that collagen is degraded sequentially from the ends by a crude clostridial collagenase preparation.

The mechanism of degradation of collagens by specific collagenases and other proteolytic enzymes is of fundamental importance to an understanding of the biochemical basis for connective tissue catabolism. It is now widely accepted that there are two types of collagenases. Bacterial collagenases, such as those from *Clostridium histolyticum* (EC 3.4.24.3), are believed to degrade collagen into small peptides by hydrolysis at multiple sites along the triple helix (Seifter & Harper, 1971). Tissue collagenases (EC 3.4.24.7), on the other hand, cleave interstitial collagens into only two fragments by action at a specific locus approximately three-fourths of the length from the N-terminus (Wooley, 1984). In spite of this major difference, it is of interest to assess whether there is any similarity between the *initial* proteolytic cleavages made by the bacterial collagenases and the single cleavage made by the tissue collagenases.

The different mode of attack of collagen by these two classes of enzymes is, no doubt, largely due to the more restrictive sequence specificity of the tissue collagenases. One important question that has yet to be resolved adequately, however, is that of the influence that collagen has in directing its own degradation. Even for tissue collagenases, sequence specificity alone is insufficient to explain their highly selective mode of attack on native interstitial collagens. The resistance of collagen to proteolysis has been attributed to its tightly coiled, triple-helical structure. Thus, the possibility exists that there are local instabilities in the triple helix or sections of presently unrecognized secondary structure that could expose certain

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