Methylation at Specific Altered Aspartyl and Asparaginyl Residues in Glucagon by the Erythrocyte Protein Carboxyl Methyltransferase*

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Protein carboxyl methyltransferases from erythrocytes and brain appear to catalyze the esterification of L-isoaspartyl and/or D-asparaginyl residues but not of normal L-aspartyl residues. In order to identify the origin of these unusual residues which occur in subpopulations of a variety of cellular proteins, we studied the in vitro methylation by the erythrocyte enzyme of glucagon, a peptide hormone of 29 amino acids containing 3 aspartyl residues and a single asparagine residue. Methylated glucagon was digested with either trypsin, chymotrypsin, pepsin, or endoprotease Arg C, and the labeled fragments were separated by high-performance liquid chromatography and identified. In separate experiments, methyl acceptor sites were determined by digesting glucagon first with proteases and then assaying purified glucagon fragments for methyl acceptor activity. Using both approaches, we found that the major site of methylation, accounting for about 62% of the total, was at the position of Asp-9. Chemical analysis of fragments containing this residue indicated that this site represents an L-isoaspartyl residue. A second site of methylation, representing about 23% of the total, was detected at the position of Asn-28 and was also shown to represent an L-isoaspartyl residue. Methyl acceptor sites were not detected at the position of Asp-15 or Asp-21. Preincubation of glucagon under basic conditions (0.1 M NH₄OH, 3 h, 37 °C) increased methylation at the Asn-28 site by 4-8-fold while methylation at the Asp-9 site remained unchanged. These results suggest that methylation sites can originate from both aspartyl and asparaginyl residues and that these sites may be distinguished by the effect of base treatment.

Protein carboxyl methyltransferase type II (EC 2.1.1.77) is a widely distributed enzyme that catalyzes the formation of methyl esters on modified aspartyl residues which occur at low levels in normal proteins (for a review, see Clarke, 1985). The function of this reaction is unknown, but it has been proposed that the methylated protein may be an intermediate in its own repair or degradation (McFadden and Clarke, 1982, 1987, Johnson et al., 1987).

Previous studies have focused on defining the chemical nature of the methyleatable aspartyl residue and its esterified product. For instance, aspartic acid β-[3H]methyl ester isolated from proteolytic digests of [3H]-methylated erythrocyte membrane and cytosolic proteins is entirely in the D-configuration (McFadden and Clarke, 1982; O'Connor and Clarke 1983, 1984). Since the maximal amount of D-aspartyl β-methyl ester from these preparations accounts for only 15% of the [3H]label, it is possible that the remaining methylated sites might represent other types of esters. One such candidate is an L-isoaspartyl α-methyl ester. L-Isoaspartyl residues have been shown in fact to be good methyl accepting substrates in a variety of synthetic peptides (Murray and Clarke, 1984; McFadden and Clarke, 1986; Johnson et al., 1987). Homologous synthetic peptides containing L-aspartyl residues are not substrates.

We were interested in this work in asking what specific residues in what protein sequences contribute to the formation of methyleatable sites. It appears that at least one source of substrate sites is L-asparagine residues. Previous work has suggested that the 39-residue polypeptide hormone ACTH could be methylated at an L-isoaspartyl-25-glycyl-26 site derived from original L-Asn-Gly residues by mild base treatment (Aswad, 1984a). Asn-Gly sequences may be particularly prone to L-isoaspartylation formation via succinimide intermediates in proteins (Bornstein and Balan, 1977; Bernhard, 1983; Blodgett et al., 1985; Geiger and Clarke, 1987). Base treatment of bovine brain calmodulin greatly increases the methyleatability of this substrate, and it has been suggested that this increase in activity may also be due to L-isoaspartyl formation from the two L-Asn-Gly sequences that are present in this protein (Johnson, et al., 1985). On the other hand, heating erythrocyte calmodulin increases its methyleatability, and this increase is independent of pH in the range 5-9 (Brunauer and Clarke, 1986). Because L-isoaspartyl formation from asparagine residues might not be expected to be independent of pH, it is possible that these heat-induced sites originate from asparaginyl residues. We were thus interested to know whether both aspartyl and asparaginyl residues could give rise to methyleatable sites.

To answer this question and to more precisely define the origin of methyleatable sites in proteins, we have chosen to study the well characterized 29-residue polypeptide hormone glucagon as a model for protein methyl acceptors. This peptide can be methylated to approximately the same extent as many red cell proteins, and because of its small size it is amenable to peptide mapping techniques (Fig. 1). In this study, we have

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The abbreviations used are: ACTH, adrenocorticotropic hormone; HPLC, high-performance liquid chromatography; [3H]AdoMet, S-adenosyl-L-[methyl-3H]methionine; SDS, sodium dodecyl sulfate.
found that the major methyl acceptor site in glucagon is derived from Asp-9 and that this site represents an L-isoaspartyl residue. A minor site of methylation occurs at an L-isoaspartyl site derived from Asn-28. Base treatment was found to enhance methylation at the Asn-28 site but cause little change in methylation at the Asp-9 site.

EXPERIMENTAL PROCEDURES

Fig. 1. Peptides of glucagon produced by digestion with pepsin (P), endoproteinase Arg C, trypsin (T), and chymotrypsin (C).

Fig. 2. Purification of 3H-methylated glucagon. Glucagon (125 μg) was incubated with [3H]AdoMet and erythrocyte protein carboxyl methyltransferase as described under "Experimental Procedures" and was separated from [3H]AdoMet and glucagon impurities by HPLC on a reverse phase column (Alltech, Econosphere C18, 4.6 × 250 mm, 5-μm spherical particles) equilibrated in 90% buffer A (0.1% trifluoroacetic acid in water) and 20% buffer B (0.1% trifluoroacetic acid, 99% acetonitrile). Glucagon was eluted from this column at room temperature at 50–58 min by a linear gradient of buffer B increasing at 1%/min to 80% B (20% A) at a flow rate of 1 ml/min. One-ml fractions were collected, and 20 μl of each fraction was counted in 10 ml of scintillation fluid. 3H-Methylated glucagon eluted at 50–53 min and [3H]AdoMet eluted at 4–8 min.

A Major Site of Methylation Is Derived from Asp-9—Glucagon was methylated with [3H]AdoMet and erythrocyte protein carboxyl methyltransferase to a maximum extent of 0.004 mol of methyl groups/mol of polypeptide, and the 3H-methylated hormone was purified by HPLC (Fig. 2). To identify the sites of methylation, 3H-methylated glucagon was digested with trypsin, the fragments were separated by HPLC, and the major UV-absorbing peaks were identified by amino acid analysis (Fig. 3). Table I; see Fig. 1 for nomenclature). All of the five major UV-absorbing peaks were found to be composed of amino acids corresponding to sequences in glucagon. These fragments resulted from tryptic cleavages after Lys-12, Arg-17, or Arg-18, except for the ArgT4 fragment which was produced by a cleavage after Trp-25. Cleavage at this position has been observed with trypsin treatment of glucagon by others (Bromer et al., 1957b; Maroux et al., 1966) and can be attributed to either residual chymotrypsin activity in the trypsin preparation or an activity inherent to trypsin (Maroux et al., 1966).

RESULTS

Fig. 3. A trypsic fragments of 3H-methylated glucagon separated by HPLC. 3H-Methylated glucagon (22.5 μg) purified by HPLC (Fig. 2) was pooled with 200 μg of unmethylated glucagon and digested with trypsin at a final concentration of 2.2 mg/ml glucagon, 0.1 mg/ml trypsin, 0.5 mg/ml calcium chloride, and 0.1 M sodium citrate at pH 6.3 for 40 min at 37°C. The incubation mixture was quenched with 10% trichloroacetic acid and the proteins were separated by HPLC as described under "Experimental Procedures." Fractions of 1 ml were collected, and 0.5 ml of each fraction was counted in 10 ml of ACS II scintillation fluid. B, methylation of tryptic fragments of glucagon. Tryptic fragments were prepared from 1 mg of glucagon and purified by HPLC as described under "Experimental Procedures." Of the 1-ml fractions collected, 0.5 ml were lyophilized and resuspended in 95 μl of 0.20 M sodium citrate, pH 6.0, in preparation for methylation assays. A portion of this material (23 μl) was incubated with 13.8 μl of erythrocyte methyltransferase isozymes and [3H]AdoMet (500 epm/μml; final concentration, 10 μM) for 1 h at 37°C. The reaction was quenched with 60 μl of 0.3 M NaOH, 1% sodium dodecyl sulfate and assayed for methyl esters as described under "Experimental Procedures." As a control, trypsin was autodigested at 0.1 mg/ml, chromatographed on HPLC, and the fractions assayed for methyl acceptor activity under the same conditions as above. No methyl acceptor activity was found in these fractions (data not shown).
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Amino acid composition of tryptic peptides of glucagon

| Glucagon 13-17 | Glucagon 1-12 | Glucagon 18-25 | Glucagon 19-29 | Glucagon 18-29 |
|---------------|--------------|---------------|---------------|---------------|
| T<sub>1</sub> | T<sub>1</sub> | T<sub>1</sub> | T<sub>1</sub> | T<sub>1</sub> |
| Axx           | 1.00<sup>a</sup> | 1.00<sup>a</sup> | 1.00<sup>a</sup> | 2.00<sup>a</sup> | 2.00<sup>a</sup> |
| Glx           | 0             | 0             | 0             | 0             | 0             |
| Ser           | 0.86          | 0             | 0.12          | 0             | 0             |
| His           | 0             | 1.13          | 0.06          | 0             | 0             |
| Gly           | 0             | 0.66          | 0             | 0             | 0             |
| Thr           | 0.06          | 2.20          | 0.08          | 0.80          | 1              |
| Arg           | 1.05          | 0.03          | 0.93          | 0.30          | 1.34          |
| Ala           | 0             | 0.80          | 0.94          | 0.97          | 0.01          |
| Tyr           | 0.41          | 0.29          | 0             | 0             | 0.86          |
| Val           | 0             | 0.14          | 0.95          | 0             | 1.10          |
| Phe           | 0             | 1.12          | 0.94          | 1              | 1.02          |
| Ile           | 0             | 0.03          | 0             | 0             | 0             |
| Leu           | 0.88          | 0             | 0             | 0             | 1.02          |
| Lys           | 0             | 1.68          | 0             | 0             | 0             |
| Trp           | 0             | 0             | 0             | 0             | 1             |

<sup>a</sup> Observed values are molar ratios with respect to Axx.

FIG. 4. Methylation of purified T<sub>1</sub> and chromatography on HPLC. The glucagon fragment, T<sub>1</sub>, HPLC purified from 200 μg of trypsin-digested glucagon, was resuspended in 150 μl of 0.2 M sodium citrate, pH 6.0, and 156 μl of this was incubated at 37 °C for 1 h with 42 μl of [3H]AdoMet (15 Ci/mmol, 1 mCi/ml in dilute H<sub>2</sub>SO<sub>4</sub> (pH 2.5–3.5):ethanol, 9:1 v:v) and 63 μl of partially purified erythrocyte carboxyl methyltransferase isozymes (see "Experimental Procedures"). This mixture was quenched by the addition of 210 μl of 10% trifluoroacetic acid, and 200 μg of trypsin-digested glucagon was added to the methylated T<sub>1</sub> as markers. This material was chromatographed on HPLC by methods identical to those used for the purification of tryptic fragments of glucagon.

None of the <sup>3</sup>H-methylated peptides comigrated exactly with any of the identified tryptic peptide-containing UV-absorbing peaks (Fig. 3A). For instance, the major peak of radioactivity migrating at 37–38 min and representing 66% of the total radioactivity eluted between T<sub>1</sub> and ArgT<sub>3</sub>'’. There are at least two reasons why a methylated peptide might not comigrate with an unmethylated fragment. First, methylatable peptides, which are likely to contain L-isosapartyl or D-aspartyl peptides, could migrate slightly differently than normal L-Asp or L-Asn-containing peptides. Furthermore, a peptide containing a methyl ester residue may be retained longer than an unmethylated peptide on reverse phase HPLC columns because of its increased hydrophobicity.

Since the addition of a methyl group was found to have a
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TABLE II
Chymotryptic peptides of glucagon

| Glucagon 26-29 | Glucagon 1-6 | Glucagon 7-13 | Glucagon 18-25 | Glucagon 14-25 | Glucagon 14-17 |
|---------------|-------------|---------------|----------------|----------------|---------------|
|               | C<sub>i</sub> | C<sub>i</sub> | C<sub>i</sub> | C<sub>i</sub> | C<sub>i</sub> |
| Asx           | 1.00<sup>*</sup> | 1             | 0.01           | 1.00<sup>*</sup> | 1             | 1.00<sup>*</sup> | 1             |
| G1x           | 0.01         | 0             | 1.00<sup>*</sup> | 0.09           | 0             | 1.00<sup>*</sup> | 2.00<sup>*</sup> | 2             | 1.00<sup>*</sup> | 1             |
| Ser           | 0.01         | 0             | 0.73           | 1.56           | 0.13          | 0.03           | 1.16           | 1.35           | 1             |
| His           | 0            | 0             | 0.72           | 0.01           | 0.03          | 0.04           | 0.04           | 0             |
| Gly           | 0.01         | 0             | 0.74           | 0.05           | 0.09          | 0.23           | 0.09           | 0             |
| Thr           | 1.03<sup>a</sup> | 1             | 0.85           | 0.67           | 0.05          | 0.04           | 0.03           | 0             |
| Arg           | 0.01         | 0             | 0.03           | 0.02           | 0.66          | 1.97           | 1.36           | 1             |
| Ala           | 0.01         | 0             | 0.01           | 0.02           | 0.72          | 1.15           | 1.15           | 0             |
| Tyr           | 0            | 0             | 0.72           | 0.05           | 1.15          | 1.15           | 0             |
| Met           | 0.87         | 0             | 0.02           | 0             | 0.02          | 0.07           | 0.16           | 0             |
| Val           | 0            | 0             | 0.10           | 0.02           | 0.83          | 1.01           | 1.01           | 0             |
| Phe           | 0            | 0             | 0.10           | 0.02           | 0.02          | 0.04           | 0.04           | 0             |
| Ile           | 0.10<sup>a</sup> | 0             | 0.01           | 0.02           | 0.02          | 0.04           | 0.04           | 0             |
| Leu           | 1.04         | 1             | 0.01           | 0.03           | 0.04          | 1.00           | 1.00           | 1             |
| Lys           | 0            | 0             | 0.01           | 0.56           | 0.04          | 0.05           | 0.15           | 0             |
| Trp           | 0            | 0             | 0              | 0              | 1             | 0              | 1             |

<sup>*</sup> Observed values are molar ratios with respect to Asx.
<sup>a</sup> Observed values are molar ratios with respect to G1x.

greater impact on peptide migration position than the isomerization or racemization of an aspararyl residue in previous studies (Murray and Clarke, 1984; 1986), we hypothesized that the radioactive peak eluting between T<sub>1</sub> and ArgT<sub>3</sub> was methylated T<sub>1</sub> peptide (glucagon 1-12). To test this hypothesis, we purified T<sub>1</sub> by HPLC from trypsin digests of unmethylated glucagon and methylated this fragment with [3H]AdoMet and erythrocyte protein carboxyl methyltransferase (cf. Fig. 3B). As shown in Fig. 4, when the methylated T<sub>1</sub> fraction was rechromatographed, a single peak of radioactivity (other than [3H]AdoMet) elutes at the same position as the large peak of radioactivity in trypsin digests of 3H-methylated glucagon (cf. Fig. 3A). As a further test, the major 3H-methylated peptide from digested glucagon was found to coelute with the product of enzymatic T<sub>1</sub> methylation when these preparations were chromatographed together (data not shown).

Based on these results, as well as the specificity of the erythrocyte protein carboxyl methyltransferase for altered asparyl residues (Clarke, 1985) and the base lability of the methyl groups on T<sub>1</sub> (96% of the radioactivity purified with 3H-methylated T<sub>1</sub> was base labile and volatile), we concluded that the Asp-9 residue in T<sub>1</sub> was the best candidate for this major methylation site in glucagon. To determine whether this site represented a racemized residue, purified T<sub>1</sub> methyl ester was analyzed for its D-aspartic content. This preparation was found to contain 1.0 mol of aspartyl residues/mol of 3H-methyl groups, and 97% of the aspartate was in the L-configuration. Because the small amount of D-aspartic acid found could be accounted for by spontaneous racemization of aspartic acid during acid hydrolysis of T<sub>1</sub>, (Manning and Moore, 1968), these data indicated that methylatable T<sub>1</sub> contains aspartate in the L-configuration. Taken together with the ability of the erythrocyte enzyme to catalyze methylation of L-isooaspartyl sites in a variety of peptides (and its failure to methylate L-aspartate residues) (Clarke, 1985; McFadden and Clarke, 1986) as well as the fact that the bulk of the glucagon preparation, which contains an L-Asp residue at position 9, cannot be methylated (see “Experimental Procedures”), these results are consistent with the hypothesis that the methylatable site in T<sub>1</sub> is an L-isooaspartate residue in the Asp-9 position.

To confirm that Asp-9 is the major methyl acceptor site in

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**Fig. 8.** Tryptic fragments of methylated base-treated and untreated glucagons purified by HPLC. Top, glucagon was base treated at a final concentration of 1.25 mg/ml in 0.1 M NH<sub>4</sub>OH at 37°C for 3 h (final pH is 10.1). This material was hydrolyzed, resuspended in 0.2 M sodium citrate, pH 6.0, and methylated with partially purified erythrocyte methyltransferases and [3H]AdoMet at final concentrations of approximately 1.2 mg/ml glucagon, 13.3 µM [3H]AdoMet (9990 cpm/pmol), and 0.16 M sodium citrate, pH 6.0, for 1 h at 37°C. This material was chromatographed by HPLC to separate [3H]AdoMet from 3H-methylated glucagon. The glucagon-containing fractions were pooled, hydrolyzed, resuspended in 0.2 M sodium citrate buffer, and digested with trypsin as described under “Experimental Procedures.” The fragments were separated by HPLC as described under “Experimental Procedures,” and 100 µl of each 1-ml fraction was counted directly in 10 ml of scintillation fluid. A radioactive peak migrating after T<sub>3</sub>, an Asp-15-containing peptide, increased by 2-fold; however, we have not been able to identify the composition of this radioactive material. In chymotryptic digests, peptides containing Asp-15 did not appear to be methyl acceptors with base treatment (data not shown). Bottom, untreated glucagon was methylated, and the 3H-methylated glucagon was purified by HPLC as above. The radiolabeled glucagon-containing fractions were pooled, hydrolyzed, resuspended in 0.2 M sodium citrate, pH 6.0, and digested with trypsin as described above. The fragments were separated as above, and 100 µl of each 1 ml fraction was counted in 10 ml of scintillation fluid.
glucagon, other proteases were used to digest \(^3\)H-methylated glucagon in experiments similar to those described above. Chymotrypsin digestion was performed as described in Fig. 5. Amino acid analyses of these peptides were consistent with glucagon chymotryptic peptides (Bromer et al., 1957a), except for two peptides (C\(_4\)\(_{\text{r}}\) and C\(_5\)) which could have been produced from glucagon by residual trypsin activity (Figs. 1 and 5, and Table II). The major peak of radioactivity eluting at 67 min on HPLC, representing about 60% of the total radioactivity, migrated several minutes after C\(_4\) (glucagon 7-12) (Fig. 5A).

Since C\(_4\) was the likely methylated species, C\(_4\) was purified by HPLC from digests of unmethylated glucagon which was enzymatically methylated with \([3\)H]AdoMet (cf. Fig. 5B). The resulting radiolabeled fragment purified by HPLC was found to migrate at the same position as the large radioactive peak from \(^3\)H-methylated glucagon digests (data not shown). Because C\(_4\) contains Asp-9 and no other possible methylation sites, this confirms the localization of the major site of methylation to Asp-9.

Similar experiments with endoprotease Arg C and pepsin digests of \(^3\)H-methylated glucagon produced radioactive fragments consistent with the results of the trypsin and chymotrypsin digestions (see the Miniprint supplement).

A second Site of Methylation Originates from the Asn-28 Site—Although it appears that most of the methylation of glucagon occurs at a site derived from Asp-9, a significant amount of \(^3\)H-methyl groups, accounting for an average of 23% of the total radioactivity in digests of \(^3\)H-methylated glucagon and about 21% of the total methyl acceptor activity in glucagon fragments, has been found in all of the experiments described above. From the evidence described below, this site appears to originate from Asn-28. In tryptic digests, a peak representing about 20% of the radioactivity migrates ahead of the ArgT\(_3\) peptide (Fig. 3A). This radioactive peak appears to represent the methyl ester of ArgT\(_3\) (see below).

In chymotryptic digests of \(^3\)H-methylated glucagon, a peak representing 21.5% of the radioactivity eluted in the region between the UV-absorbing peaks of C\(_4\) (glucagon 1-6) and C\(_2\) (glucagon 7-13) (Fig. 5A). Although from the labeled peak's migration position it might be considered C\(_4\) methyl ester, this seemed unlikely because C\(_4\) contains no aspartate or asparagine. A likely candidate for the parent of this methyl ester was the tetrapeptide, C\(_3\), which could be prone to L-isoaspartyl formation at Asn-28 (Johnson et al., 1985). Since the tryptic peptide ArgT\(_3\) contains this site as well, it appeared that in this peptide Asn-28 was the site of methylation and not Asp-21.

In order to confirm that the radioactive peak from trypsin digestion designated as ArgT\(_3\) methyl ester did indeed contain a methylation site at the Asn-28 position, the radioactive peak eluting at 52-53 min (Fig. 3A) was isolated and digested further with chymotrypsin. This treatment should result in the formation of C\(_4\) methyl ester. Upon digestion of the putative ArgT\(_3\) methyl ester peptide and HPLC we found a radiolabeled peak migrating between C\(_4\) and C\(_5\) in the position of C\(_4\) methyl ester (data not shown). No radiolabel was found in a region expected of C\(_4\) methyl ester suggesting that no methylation occurred at Asp-21.

To test methylation at the Asn-28 site more directly, we decided to isolate a peptide containing Asn-28 and show that such a peptide could be a methyl acceptor. Unfortunately, none of the UV-absorbing peaks containing Asn-28 peptides were methylatable. Instead, the methylatable fractions which contained about 20% of the methyl acceptor activity appeared to migrate just ahead of the Asn-28-containing peptide peaks. In methylation of tryptic fragments, a small amount of methyl acceptor activity migrates just ahead of ArgT\(_3\) (Fig. 3B). In methylation of chymotryptic fragments, methyl acceptor activity elutes 10 min ahead of C\(_4\) (Fig. 5B). It appears that these fractions, probably containing L-isoaspartic acid or D-aspartic acid, migrate ahead of their counterparts containing asparagine. The methylatable chymotryptic fragment eluting ahead of C\(_4\) was found to elute in the position expected for C\(_4\) methyl ester when it was enzymatically \(^3\)H-methylated and rechromatographed (see above).

To demonstrate that the methylatable C\(_4\) peptide could be derived from Asn-28, we employed the base treatment technique of Johnson et al. (1985) which appears to produce methylatable sites from asparaginyl, probably by promoting L-isoaspartyl formation. Thus, we isolated the unmethylated peptide C\(_4\) (glucagon 26-29) from a chymotryptic digest of glucagon, treated it with 0.1 M ammonium hydroxide at 37 °C, and tested it for methyl acceptor activity. The base-treated C\(_4\) was found to be a methyl acceptor whereas untreated C\(_4\) was not. Furthermore, when the methylated base-treated C\(_4\) was rechromatographed with chymotryptic peptide markers, it was found that the methylated species migrated in the same position as the C\(_4\) methyl ester previously described.

Identification of the Methylation Residue Originating from Asp-9 and Asn-28 as L-Isoaspartate—The results described above are consistent with the assignment of the Asp-9 and Asn-28 sites of methylation with L-isoaspartyl residues. To directly test these assignments, we chemically synthesized authentic standards of peptides corresponding to the chymotryptic peptides C\(_2\) and C\(_3\) but with the substitution of an L-isoaspartyl residue for the normal L-aspartyl and L-asparaginyl residues, respectively. These peptides were found to be stoichiometrically methylated (1.2 and 0.79 mol of methyl groups/mol of peptide) by the erythrocyte methyltransferase under the same conditions that the C\(_4\) and C\(_3\) peptides derived from glucagon as well as intact glucagon were substoichiometrically methylated (less than 0.01 mol of methyl groups/mol of glucagon or peptide) (see “Experimental Procedures”). If the methylation sites in \(^3\)H-methylated glucagon were at L-isoaspartyl residues, we would expect that the methylated synthetic and natural peptides would be identical. We tested this possibility by HPLC. When the synthetic isoC\(_2\) peptide was enzymatically methylated and chromatographed with chymotryptic peptides of glucagon the synthetic peptide ester was found to elute at the same time as the C\(_2\) ester from chymotryptic-digested \(^3\)H-methylated glucagon (data not shown; cf. Fig. 5A). Each of these peaks was methylated and co-chromatographed on the same HPLC system a single peak of radioactivity was found. Similarly, when the synthetic isoC\(_3\) peptide was methylated and chromatographed on HPLC, the peptide ester eluted in the same position as C\(_3\) ester from chymotryptic-digested \(^3\)H-methylated glucagon (data not shown; cf. Fig. 5A). Each esterified C\(_4\) peptide, either isolated from the methylation of the synthetic isoC\(_4\) peptide or digestion of \(^3\)H-methylated glucagon, was found to spontaneously produce a radiolabeled degradation product migrating 6 min earlier than the parent ester peak on HPLC. Both the parent ester and the new radiolabeled peak from both the synthetic peptide and \(^3\)H-methylated glucagon were found to co-migrate on HPLC.

Site of Methylation in Synthetic Glucagon—All of the studies above were performed with glucagon isolated from bovine and porcine pancreas. To determine whether the isoaspartyl groups at positions 9 and 28 were generated during the biosynthesis, cellular processing, or purification of glucagon, we enzymatically methylated a sample of glucagon, containing...
the same amino acid sequence, but which had been chemically synthesized (see "Experimental Procedures"). We found that the degree of methylation of this material was similar to that of the natural sample. Tryptic digestion of \(^{3}H\)-methylated synthetic glucagon under the conditions used in Fig. 3 revealed that the bulk of the radioactivity (87%) was associated with the Asp-9-containing fragment; no radioactivity was detected in the fragment containing the Asn-28 site. These results indicate that the formation of an isoaspartyl residue at Asp-9 does not require cellular processes and may reflect an inherent chemical instability of this isoaspartyl residue. On the other hand, these data suggest that this is not the case for the asparagine 28 residue.

**Base Treatment of Glucagon Increases Methylation at Asn-28 but Does Not Affect Methylation at Asp-9**—It has been suggested that base treatment of proteins enhances their methyleatability by catalyzing L-isoaspartyl formation from asparagines (Johnson et al., 1985). As shown above, base treatment of C₁ (L-Leu-L-Met-L-Asn-L-Thr) leads to the production of methyleatable peptide. We were interested to know whether this treatment could lead to the production of methyleatable sites from aspartyl residues such as Asp-9, the major methyl acceptor in native glucagon. Therefore, glucagon, which was base treated under the conditions described by Johnson et al. (1985) was methylated with \(^{3}H\)AdoMet and chromatographed by HPLC. There was no difference between the UV profiles of the base-treated and untreated glucagons, while there was a significant difference between the radiolabel profiles of these two samples (Fig. 8). The major radioactive peak in the base-treated glucagon digest was Arg\(_T\) \(_T\)-methyl ester, an Asn-28-containing peptide. The amount of Arg\(_T\) \(_T\) methyl ester per mol of glucagon increased 6-fold over that amount in methylated untreated glucagon. Similarly, an increase in the amount of radiolabel with Thy methyl ester, another Asn-28-containing peptide, is observed. However, no change in the amount of radiolabel with Thy methyl ester (an Asp-9-containing peptide) is seen. Similarly, in chymotrypsin digests of methylated base-treated glucagon a large increase in the amount of Asn-28-containing peptide, C₁ methyl ester, is observed while no change in the amount of C₁ methyl ester (an Asp-9-containing peptide) is seen (data not shown). These data indicate that in whole glucagon, as well as in the C₁ peptide, base treatment produces methyleatable substrates by altering the Asn-28 site. However, these conditions did not enhance methylation at the Asp-9 site.

**Methylation Does Not Appear to Occur at Sites Originating from Asp-15 or Asp-21**—Methylation of sites derived from Asp-9 and Asp-28 together accounts for about 85% of the total methylation of glucagon. The remainder of the methylation does not appear to be derived from the other two possible origins of methyleatable sites, Asp-15 or Asp-21, since peptides containing these sites do not appear to be methyl acceptors (cf. Figs. 3B and 5B–7B) and peptides containing these sites produced by digestions of enzymatically \(^{3}H\)-methylated glucagon do not appear to be associated with radiolabel (cf. Figs. 3A and 5A–7A). In any case, if methylation occurred at either of these sites they could account for a maximum of 15% of the total methylation of glucagon.

**DISCUSSION**

Although it is now clear that widely distributed type II protein carboxyl methyltransferases catalyze the methyl esterification of unusual D-aspartyl and L-isoaspartyl residues, the origin of these residues in cellular proteins has not been established (Clarke, 1985). Previous work has indicated that base treatment of the peptide hormone ACTH can generate a methyleatable L-isoaspartyl residue from the asparagine residue in position 25 (Aswad, 1984a), but the situation for other proteins and peptides, especially those studied under physiological conditions, is not known. The mechanisms of isoaspartyl formation in an asparagine residue have been proposed to be the hydrolysis of a succinimide intermediate formed in an intramolecular attack of the peptide nitrogen atom on the side chain carbonyl carbon. The rate of this reaction is dependent upon the bulk of the side chain of the adjoining residue on the carboxyl side. Maximal succinimide formation is found when there is little or no steric hindrance from this group, such as in an Asn-Gly sequence (Bornstein and Balian, 1977; Aswad, 1984a; Murray and Clarke, 1984; Blodgett et al., 1985; Geiger and Clarke, 1987).

An analogous reaction has been shown to occur with aspartyl residues in model peptides, but at a much slower rate. For example, the rate of isoaspartyl formation from Val-Tyr-Pro-Asp-Gly-Ala is about 38 times slower than that for the corresponding Asn-containing peptide at pH 7.4, 37°C (Geiger and Clarke, 1987). From these considerations, one might predict that the order of the rates of isoaspartyl formation in glucagon might be Asn-28-Thr-29, followed by Asp-15-Ser-16, and then by Asp-9-Tyr-10 and Asp-21-Phe-22. Although in this work we did find that a small fraction of the methyleatable sites was derived from the Asn-28 residue, the major site was unexpectedly found to be at the Asp-9 residue. It does not appear that isoaspartyl residues did form at other sites but were not recognized by the methyltransferase because all peptides tested to date containing L-isoaspartyl residues have been shown to be substrates (Murray and Clarke, 1984; McFadden and Clarke, 1986; Johnson et al., 1987; cf. "Results"), and we do not observe any methylation of glucagon fragments containing Asp-15 or Asp-22 residues (cf. Figs. 3B and 5B). Our results suggest that isoaspartyl residue formation in proteins may not be strictly limited to asparagine-glycine sequences or even to asparagine residues.

**Intrinsic Structural Features of Glucagon Appear to Account for L-Isospartyl Formation at Asp-9**—Why should isoaspartyl residues occur preferentially at Asp-9 in glucagon? We have considered the possibility that this abnormal residue is generated by an error in the biosynthesis of the hormone precursor (cf. Clarke, 1985) or by chemical modification at this site during the cellular processing of glucagon precursor. However, our observation that Asp-9 is preferentially isomerized in chemically synthesized glucagon as in glucagon isolated from pancreatic tissue indicates that there is an instability inherent in the chemical structure of glucagon that may enhance isoaspartyl formation at Asp-9. Assuming that a succinimide is involved, this formation could be explained by intramolecular interactions that result in an increase in the nucleophilia of the Tyr-10 peptide nitrogen or an increase in the susceptibility of the Asp-9 carbonyl carbon to attack or an increase in the leaving group potential of the Asp-9 \(\gamma\)-hydroxyl group. It is of interest to note that the tyrosine residue at position 10 has an unusually low pKₐ value and that this region of the polypeptide appears to have multiple interactions in the binding of glucagon to its receptor (Krestenansky et al., 1986).

Our results suggest that the identification of methylation sites may be more complex than we originally anticipated and that other factors such as the interaction of aspartic acid or asparagine residues with neighboring groups brought into proximity with these sites by the three-dimensional structure of the peptide or protein may be crucial in determining which sites will ultimately be degraded to isoaspartyl residues and...
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then recognized by the cellular methyltransferases.

Base Treatment Enhances Methylation at Sites That Orig-inate from Asparagine Residues but Does Not Affect Methyla-
tion at Aspartate-derived Sites—In this study we have used glucagon as a model peptide to determine the effects of mild base treatment on the methylibatability of sites derived from asparagyl and aspartyl residues. We have found that the methylibatability of the Asn-28 site was increased following base treatment. However, methylation at the Asp-9 site, the major methyl acceptor site in untreated glucagon, was unchanged by base. Asp-15 and Asp-21 did not appear to be affected by base because we found no evidence that either became a methyl acceptor upon base treatment.

These data indicate that base does not appear to catalyze the formation of methylatable residues from aspartyl residues. If isoaspartates are produced from aspartates via succinimide formation, the increase in the nucleophilicity of the peptide bound N-H may be balanced by the deprotonation of the p-carboxy group of aspartate. Basic conditions may also reduce the capacity of other protonated groups to stabilize the p-carboxy group of aspartate during succinimide formation. In the base-enhanced formation of methylatable residues from asparagine, the increased nucleophilicity of the peptide bond N-H appears to outweigh any reduction in the leaving group potential of the asparagine -NH2. Therefore, base treatment could be a useful experimental method to distinguish between methylation sites arising from aspartyl and asparaginyl residues. The usefulness of this method has been confirmed in preliminary studies using synthetic asparagine- and aspartic acid-containing peptides.

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SUPPLEMENTARY MATERIAL TO: METHYLATION AT SPECIFIC ALTERED ASPARTY AND ASPARAGINYL RESIDUES IN GLUCAGON

BY THE ORTHOXANTHINE PROTEIN CATALYZER METHYLTRANSFERASE

Irene M. Ota, Li Ding, and Steven Clarke

Experimental procedures

Proteolytic digestions of glucagon — Glucagon, obtained as crystals from bovine and porcine pancreases (Sigma), is soluble at room temperature to a maximum concentration of about 50 μg/ml in the pH range 1.0-5.5 (Staun et al., 1955). In order to prepare soluble solutions of glucagon at concentrations of 3.25-5.0 μg/ml in 0.2 M sodium citrate, pH 6.0 for proteolytic digests, glucagon was dissolved by heating in a boiling water bath for about 15 minutes. This solution was then cooled to 37ºC and the appropriate proteases were added immediately.

Trypsin (Sigma, specific pancreas, treated with epsilon carbamyd chloride, 1000-5000 Kunitz-trypsin units/mg water (KUP) soluting of protein) was dissolved in 0.01 M HCl to a protein concentration of 1 mg/ml and typically 4 mg of this solution was incubated with 10 μl of a 1.5 mg/ml solution of glucagon (prepared as above) and 16 μl of 5 mg/ml CaCl2 in a 1.9 ml polyethylene tube at 37ºC for 40 minutes. After this time, the reaction was quenched with 100 μl of 10% w/v trichloroacetic acid (TCA), (pellet, supernatant grace). Crystamylase (Sigma, bovine pancreas) 1.5 mg/ml was dissolved in 0.2 M sodium acetate (pH 5.0) and added to the above solution of TCA. After 1 hour, the reaction was quenched with 100 μl of 10% w/v trichloroacetic acid (TCA).

Tryptic phosphopeptides (from 200 μg to 1 mg of digested glucagon) were applied to a 10x10 cm column equilibrated in 100 mM buffer B (50 mM buffer B) at 4 °C. Both 10x10 cm columns were equilibrated in 0.7 M buffer B and 2.5 M buffer B. The overlapping samples were separated similarly. Crystamylc peptides (12.5 μg) were separated as above except that a concave gradient (gradient A 0.5 M in a 5 min. pH 4.8, 0.5 M in the next 35 min.) was used. Enzyme-linked Arg-lysine and Arg-proline-labeled fractions were applied to a column equilibrated in 100 mM buffer B (50 mM buffer B) and were eluted with a linear gradient increasing to 10% B (2.5 M) in 40 minutes. The fractions were collected and the absorbance at 214 nm was monitored by a Waters 410 UV detector.

HPLC fractions containing glucagon peptides detected by their absorbance at 214 nm were pooled and lyophilized. These samples were resuspended in 0.5 M in 1 ml of water obtained from a Millipore filtration system (Barnstedt Sybron Corporation, high specific resistance: 10-10 cm/s). The reconstituted samples, ranging in concentration from 5 mg/ml to 1 mg/ml, were transferred to a 50 ml borosilicate glass tubes, then lyophilized and hydrolyzed in same as a 6 N HCl vapor using a Waters HPLC work station (Belmaneppe et al., 1984). Up to 10 hydrolysates tubes were placed in a glass vial with 200 μl of 6 N HCl (interfer, sesquial glycolic acid and sodium chloride, and sealed with a filled cap. The water was sealed alternately to a vacuum or a stream of nitrogen until the vial was purged. The sealed evacuated vial was then placed in a 150°C oven for 1 h. Residual HCl was removed by lyophilization.

For tryptic-generated glucagon fragments a different hydropysis procedure was used. The lyophilized HCl-vials containing glucagon fragments were digested in 100 μl of 6 N HCl (interfer, sesquial glycolic acid and sodium chloride) and reconstituted in 200 μl of 6 N HCl (interfer, sesquial glycolic acid) at 100 °C. The samples were then lyophilized and reconstituted in appropriate amounts of NaOH water containing approximately 50 mg of amino acids. After rehydration at room temperature for 1 h, 15% of the samples were applied to a C18 reverse phase column (Waters Resolve, 30 μM x 15 cm, 5 μm spherical
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Methylation of Glucagon Fragments — Peptides were assayed for their methyl acceptor activity after incubating them with hydroxyproline (carboxy methyl)transferase and S-adenosyl-L-[methyl-3H]methionine. The amounts of radioactivity transferred to methyl esters was quantitated by a method developed after base hydrolysis.

Peptides collected from HPLC were lyophilized and resuspended in 0.2 M sodium citrate, pH 6.0, to a concentration of 2 mg/ml. The solutions were incubated at 37°C for 20 min with 15 μl of a mixture of enzymes partially purified by affinity chromatography on a column of glutamic acid-Sepharose 4B. At the end of the incubation the reaction mixtures were washed on a column of S-adenosyl-L-[methyl-3H]methionine-Sepharose 4B (20 ml), dialyzed against 100 mM sodium acetate buffer, pH 7.5, and lyophilized. The samples were assayed for their methyl acceptor activity after incubation with 0.2 M sodium citrate, pH 6.0, for 15 min at 37°C. The methylated peptides were then separated by high performance liquid chromatography (HPLC) on a column of TSKgel ODS-80, 2.5 μm, 25 cm × 0.46 cm. The elution buffer was 0.1 M sodium acetate, pH 6.0, and the elution was done isocratically with a linear gradient of methanol.

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Figure 6. (A) Endoprotease Arg C digestion of 3H-methylated glucagon and the separation of fragments by HPLC. 3H-Methylated glucagon was purified as described in Fig. 2 and approximately 24.5 µg was pooled with 42.1 µg of unmethylated glucagon and digested at a final concentration of 0.44 µg/ml glucagon, 0.05 µg/ml endoprotease Arg C and 0.2 M sodium citrate, pH 6.00 at 37°C for 20 min. This mixture was quenched with 250 µl of 10% TFA and chromatographed as described in Experimental Procedures. Fractions of 1 ml were collected and 0.5 ml was counted in 10 ml of scintillation fluid. (B) Endoprotease Arg C fragments purified on HPLC and methylated with erythrocyte protein carboxyl methyltransferase and [3H]AdoMet. Endoprotease Arg C fragments were prepared from 156 µg of glucagon digested at a final concentration of 0.63 µg/ml glucagon, 0.05 µg/ml endoprotease Arg C and 0.2 M sodium citrate, pH 6.0 at 37°C for 20 min. The mixture was quenched with 250 µl of 10% TFA and chromatographed on HPLC as described in Experimental Procedures. Fractions of 1 ml were collected, lyophilized and reconstituted in 60 µl of 0.2 M sodium citrate, pH 6.0 for methylation assays. Of this solution, 30 µl was incubated with 16 µl of a mixture of the protein carboxyl methyltransferase isozymes and [3H]AdoMet (200 cpmlpmol) at a final concentration of 30 µM for 1 h at 37°C and was quenched with 60 µl of 0.6 M sodium borate/15 SDS, pH 10.7. Of this mixture, 60 µl was assayed for methyl groups as described in Experimental Procedures.

Table III.

Endoprotease Arg C Fragments of Glucagon

| Gluc. 1-18 | Gluc. 19-29 |
|------------|------------|
|            | abs. theor. abs. theor. |
| Ala        | 2.00 ± 1 2.00 ± 2 |
| Glx        | 1.00 ± 2 1.00 ± 1 |
| Ser        | 0.50 ± 4 0.50 ± 4 |
| His        | 0.25 ± 3 0.25 ± 3 |
| Gly        | 0.00 ± 1 0.00 ± 1 |
| Thr        | 0.97 ± 2 0.97 ± 2 |
| Arg        | 0.61 ± 3 0.61 ± 3 |
| Ala        | 0.99 ± 2 0.99 ± 2 |
| Tyr        | 0.99 ± 0 0.99 ± 0 |
| Met        | 0.01 ± 1 0.01 ± 1 |
| Val        | 0.12 ± 1 0.12 ± 1 |
| Phe        | 0.30 ± 2 0.30 ± 2 |
| Ile        | 0.32 ± 0 0.32 ± 0 |
| Leu        | 0.95 ± 1 0.95 ± 1 |
| Lys        | 0.01 ± 0 0.01 ± 0 |
| Trp        | 0.01 ± 0 0.01 ± 0 |

Observed values are molar ratios with respect to Ala.
Evidence for a minor methylated site at Asn-28 was obtained from pepsin, endoproteinase Arg C and pepsin digests supporting the results from tryptic and chymotrypsin digestion described in the "Results" section. For example, in endoproteinase Arg C digests of 3H-methylated glucagon we detect a peak accounting for 9% of the total radioactivity that migrates slightly ahead of the T5 peptide (glucagon 19-27) (Fig. 6A). Similarly, two peaks of radioactivity, representing 75 and 25% of the total methylation, elute after T5p and P3, which are two related peptides containing only Asn-28 as a likely site for methylation (Fig. 7A). From these results, it appears that methylated peptide elutes slightly before the asparagine-containing peptide.

Table IV: Peptidic Fractions of Glucagon

| Gluc. 1-9 | Gluc. 22-29 | Gluc. 23-29 | Gluc. 26-27 | Gluc. 29-29 | Gluc. 19-22 | Gluc. 1-21 |
|----------|-------------|-------------|-------------|-------------|-------------|-------------|
| obs. theor. | obs. theor. | obs. theor. | obs. theor. | obs. theor. | obs. theor. | obs. theor. |
| 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 1.10 | 1.10 | 1.10 | 1.10 | 1.10 | 1.10 | 1.10 |
| 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |

Observed values are molar ratios with respect to Asn. Observed values are molar ratios with respect to Asn. Some newspaper may have been lost during encoding. Approximately 50% of the material may be due to a contaminant.