Mammalian protein carboxyl methyltransferases have recently been proposed to recognize atypical configurations of aspartic acid and may possibly function in the metabolism of covalently altered cellular proteins. Consistent with this proposal, the tetrapeptide tetragastrin, containing a single "normal" L-aspartyl residue (L-Trp-L-Met-L-Asp-L-Phe-NH₂) was found here not to be an in vitro substrate for erythrocyte carboxyl methyltransferase activity. However, chemical treatment of tetragastrin by methyl esterification and then de-esterification of the aspartic acid residue yielded a mixture of peptide products, the major one of which could now be enzymatically methylated. We show here that this new peptide species is the isomeric D-aspartyl form of tetragastrin (L-iso-tetragastrin; L-Trp-L-Met-L-Asp-L-Phe-NH₂), and it appears that isomerization proceeds via an intramolecular succinimide intermediate during the de-esterification procedure. L-iso-Tetragastrin is stoichiometrically methylated (up to 90% in these experiments) with a Kₐ for the enzyme of 5.0 μM. Similar chemical treatment of several other L-aspartyl peptides also resulted in the formation of new methyltransferase substrates. This general method for converting normal aspartyl peptides to isoaspartyl peptides may have application in the reverse process as well.

Recent studies of the substrate specificities of enzymatic protein carboxyl methylation reactions have helped to define the functions of these reactions in cells (Clarke, 1985). For example, analysis of the site-specific methylation of a set of L-glutamic acid residues in bacterial chemoreceptor proteins has led to a detailed model for the regulation of receptor function through reversible covalent modification of these sites (Terwilliger and Koshland, 1984; Kehry et al., 1983; Boyd et al., 1983; Stock et al., 1985).

A very different type of protein carboxyl methylation reaction, studied mainly in mammalian cells, appears to occur only at aspartic acid sites in nonconventional configurations. This conclusion is based on the recovery of D-aspartic acid β-methyl ester from proteolytic digestions of radioactively methylated erythrocyte proteins and on the nitro methylation of a synthetic peptide related to adrenocorticotropic but containing a β-isomerized L-aspartyl residue (L-Tyr-L-Val-L-Pro-L-Asp-Gly-L-Ala) (McFadden and Clarke, 1982; O'Connor and Clarke, 1983, 1984; Aswad, 1984a; Murray and Clarke, 1984). To explain this unusual enzyme specificity, several related models have been proposed suggesting that this protein carboxyl methyltransferase, which had previously been characterized and purified on the basis of its substoichiometric recognition of a wide range of proteins (Paik and Kim, 1980), is in fact a protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase which recognizes these covalently altered sites that may arise spontaneously in many proteins.

The possible cellular functions of this enzymatic methylation reaction have also been discussed (Clarke, 1985). In one such model, the enzymatic methyl esterification of altered protein sites leads to further covalent rearrangements of these sites, resulting in their ultimate conversion back to "normal" L-aspartyl or L-asparaginyl sites in the cell. A proposed intermediate in this process of rearrangement, a five-membered succinimide ring, has been shown in model studies to form rapidly when enzymatically methylated peptides related to ACTH undergo spontaneous demethylation at physiological pH (Johnson and Aswad, 1985; Murray and Clarke, 1986). Hydrolysis of the succinimide then leads to the formation of a mixture of isoaspartyl and normal aspartyl residues. In a sense then, the enzymatic methylation "activates" the site, lowering the barrier to the formation of the ring intermediate and to further rearrangements.

We considered that this proposed activation and rearrangement of aspartyl sites in cells might be simply modeled by the methylation and demethylation of aspartyl peptides using strictly chemical procedures. We report here that the peptide tetragastrin (L-Trp-L-Met-L-Asp-L-Phe-NH₂) can be covalently rearranged by chemical methyl esterification and de-esterification and that a rearranged form of this peptide, L-iso-tetragastrin, is a substrate for erythrocyte protein carboxyl methyltransferase. This chemical procedure thus converts a normal aspartyl peptide to an isoaspartyl peptide and is effectively the reverse of a "repair" reaction. Using this convenient approach, we have converted several additional commercially available peptides into substrates for erythrocyte protein carboxyl methyltransferase.
Esters of aspartyl residues in synthetic polypeptides are characteristically labile due to the favorable intramolecular formation of succinimide derivatives (Bernhard, 1983; Johnson and Aswad, 1985; Murray and Clarke, 1986). This mechanism of intramolecular ester cleavage can also explain the rapid rates of demethylation at physiological pH of enzymatically formed protein methyl esters (Terwilliger and Clarke, 1981; Barber and Clarke, 1985). Hydrolysis of peptide succinimides can occur at both the \( \alpha \)- and \( \gamma \)-carboxyl groups; this results in the formation of a mixture of normal and isomerized peptides. We decided to study the chemistry of methylation-induced aspartyl rearrangements using the tetrapeptide tetragastrin as a model peptide (Fig. 1).

![Fig. 1. Proposed chemical reactions for the covalent rearrangement of L-aspartyl peptides. Tetragastrin, in its normal form, contains the single free \( \beta \)-carboxyl group of Asp-3. This carboxyl group can be methyl esterified by treatment with acidic methanol, and subsequent displacement of the ester group by the neighboring amide nitrogen of Phe-4 results in the formation of an intramolecular five-membered succinimide ring. Ring opening by hydronium ion attack at the \( \alpha \)-carboxyl position leads to an isomerized peptide bond between Asp-3 and Phe 4. Ring opening at the \( \beta \)-position yields a normal peptide configuration identical to the starting material. If the intermediate succinimide peptide is prone to racemization at the aspartyl center (Clarke, 1985), then the final mixture of peptides may also contain \( \delta \)-(L-aspartyl)tetragastrin in the normal and iso configurations.](image)

**RESULTS**

Esters of aspartyl residues in synthetic polypeptides are characteristically labile due to the favorable intramolecular formation of succinimide derivatives (Bernhard, 1983; Johnson and Aswad, 1985; Murray and Clarke, 1986). This mechanism of intramolecular ester cleavage can also explain the rapid rates of demethylation at physiological pH of enzymatically formed protein methyl esters (Terwilliger and Clarke, 1981; Barber and Clarke, 1985). Hydrolysis of peptide succinimides can occur at both the \( \alpha \)- and \( \gamma \)-carboxyl groups; this results in the formation of a mixture of normal and isomerized peptides. We decided to study the chemistry of methylation-induced aspartyl rearrangements using the tetrapeptide tetragastrin as a model peptide (Fig. 1).

**FORMATION OF A METHYL-ACCEPTING PEPTIDE BY CHEMICAL TREATMENT OF TETRAGASTRIN**—Protein carboxyl methyltransferases from brain and erythrocytes apparently do not recognize normal L-aspartyl peptides as substrates (Murray and Clarke, 1984; Aswad, 1984a), and this was found to be the case here with normal tetragastrin. Untreated commercial tetragastrin was not detectably methyl esterified by carboxyl methyltransferase activity in crude erythrocyte cytosol (Fig. 2). However, preliminary experiments indicated that simply by chemically esterifying tetragastrin and then treating this methyl ester with mild base, we could form material which could now be methylated by enzyme activity in erythrocyte cytosol (Fig. 2).

We performed control experiments to determine whether both the esterification and de-esterification treatments are necessary to form enzyme substrate. As shown in Fig. 2, acidic treatment (pH 2) similar to that of the esterification step did not lead to the formation of methyl acceptor substrate. Alkaline pH treatment alone, as in the de-esterification step, did generate a small level of enzyme substrate (Fig. 2). However, later experiments indicated that the commercial tetragastrin HCl contained a 1% contaminant of succinimide, and alkaline treatment of this imide may be responsible for the formation of a low amount of enzyme substrate. Mild alkaline treatment of purified tetragastrin produced no detectable enzyme substrate.
Peptide Substrates for Protein Carboxyl Methyltransferase

FIG. 3. Chromatographic separation of purified tetragastrin and tetragastrin methyl ester. The trifluoroacetate salts (2.2 nmol, upper trace) and tetragastrin methyl ester (1.7 nmol, lower trace) were analyzed by the standard conditions of reversed phase liquid chromatography described under “Materials and Methods.” The amount of material was determined by amino acid analysis as in Table I. Analysis of the peak areas allows us to calculate an extinction coefficient at 214 nm of 35,000 M⁻¹ cm⁻¹ (± 2000).

TABLE I

Amino acid composition, D/L-aspartate analysis, and mass spectra of peptides

Peptides were purified by HPLC and recovered as lyophilized trifluoroacetate salts. Approximately 1 nmol was acid hydrolyzed, and a portion of the dried residue was analyzed by the orthophthalaldehyde method described under “Materials and Methods.” Compositions were normalized to the aspartic acid content. D/L-Aspartate ratios were determined as described under “Materials and Methods.” Fast atom bombardment positive ion mass spectrometry was performed on a modified Kratos MS9 instrument operated by Dr. Dilip Sensharma at the UCLA Department of Chemistry and Biochemistry. Samples (approximately 10–50 µg) were suspended in 20 µl of glycerol and were applied to a room temperature stainless steel electrode that was bombarded by xenon atoms emitted at a gun voltage of 4–6 kV and a current of 1 mA. Parent ion masses are indicated for protonated (M+1) and sodium species (M+23); values in parentheses are the expected masses for these positive ions. Not shown are the masses of prominent fragment ions which were consistent with the expected peptide sequences (Schafer, 1983).

| Peptide        | Asp | Met | Phe | Trp | Asp n/L ratio | Parent ion mass |
|----------------|-----|-----|-----|-----|--------------|----------------|
| Tetragastrin   | 1.00| 0.90| 1.03| 1.00| 0.050        | 597 (597)       |
| Tetragastrin   | 0.90| 0.95| 1.14| 1.11| 0.111        | 611 (611)       |
| methyl ester   |     |     |     |     |              | 633 (633)       |
| L-iso-Tetragastrin | 1.00| 0.91| 1.00| 1.02| 0.042        | 597 (597)       |
|                |     |     |     |     |              | 619 (619)       |

* Tryptophan, destroyed by acid hydrolysis, was determined by UV absorbance measurements of the intact peptides, with calculations based on the extinction coefficient of free tryptophan (see also Table II).

We next followed a standard protocol (Kim et al., 1983) to partially purify erythrocyte carboxyl methyltransferase activity, using esterified/de-esterified tetragastrin as the methyl acceptor in the enzyme assay. It was found that during ammonium sulfate precipitation and gel filtration chromatography, the peptide-methylating activity behaved identically with protein carboxyl methyltransferase activity (assayed by the methylation of ovalbumin). We conclude that the activity that recognizes esterified/de-esterified tetragastrin is the same as the well-characterized erythrocyte protein carboxyl methyltransferase (Kim, 1974; Kim et al., 1983).

Tetragastrin, purified to homogeneity by reversed phase HPLC (Fig. 3), was converted to the β-methyl ester with acidic methanol. The esterification reaction, performed on a 1.6-µmol scale as described under “Materials and Methods,” was 28% complete after 1 h, 48% complete after 2 h, and 98% complete after 19 h as measured by HPLC. No products other than the starting material and the ester were detected at time 26 h.

FIG. 4. Hydrolysis of tetragastrin methyl ester at pH 9.5 and enzymatic methylation of the product peptides. Tetragastrin methyl ester (50 µM in 1 mM HCl) was added to an equal volume of 1 M NH₄ acetate, pH 9.5, and incubated at 23 °C. At various times, aliquots representing 0.5 nmol of starting ester material were quenched by 1 volume of 1 M acetic acid and then analyzed by the standard conditions of reversed phase HPLC described under “Materials and Methods.” Elution positions are indicated for tetragastrin methyl ester, tetragastrin imide, L-iso-tetragastrin, and normal tetragastrin. For the final time point (26 h), an aliquot equivalent to 15 nmol of starting ester material was quenched by 1 M acetic acid, and the solvent was removed by lyophilization before HPLC analysis. The peptides eluted relatively early in this final chromatogram (26 h) due to the larger sample load. Several unidentified peaks and shoulders (*) were reproducibly present and may include the D-aspartyl derivatives of iso-tetragastrin and tetragastrin which have been found to migrate after the corresponding L-derivatives (P. N. McFadden and S. Clarke, manuscript in preparation). Fractions (1 ml) from the 26-h chromatogram were lyophilized, and the residual material was tested for the presence of methyl-accepting peptides by incubation (37 °C) with 100 µl of a mixture containing 0.5 mg of erythrocyte cytosol protein and 2 nmol of [methyl-3H]-S-adenosylmethionine (440 dpm/pmol) in a final buffer composition of 140 mM sodium citrate, 1.5 mM sodium phosphate, 1.5 mM Na₂-EDTA, 4.5 mM β-mercaptoethanol, 3% glycerol, pH 6. The enzymatic incubations were quenched after 30 min by 100 µl of 0.2 M NaOH, 1% sodium dodecyl sulfate. The graph at the top of the figure shows the resulting enzymatic carboxyl methylation, determined as [3H]methanol with the vapor diffusion assay.
Peptide Substrates for Protein Carboxyl Methyltransferase

I  

pH 7.4 Ester Hydrolysis

220 min

84 min

40 min

0.1 A

Ester

Normal

L-iso

22 h

Peptide  Substrates for Protein Carboxyl Methyltransferase

points up to 2 h; after 19 h a third unidentified peak, migrating about 10 min later than the ester peak on HPLC, accounted for 9% of the UV absorption. Following the 19-h reaction, the tetragastrin methyl ester was purified by HPLC (Fig. 3), and the structure of the ester was confirmed by its amino acid composition and its molecular mass (Table I).

When tetragastrin methyl ester was incubated in a pH 9.5 buffer at 23 °C, several new peptide species formed (Fig. 4). A new peak of material, tentatively identified as peptide succinimide, formed at early times and then gradually disappeared. Two other major new peaks formed and were stable; one of these eluted identically as normal L-tetragastrin, and the other was tentatively identified as L-iso-tetragastrin. The higher yield of the isomerized species, formed at four times the level of the normal peptide, was consistent with previous patterns of succinimide cleavage (Bernhard, 1983; Johnson and Aswad, 1985; Murray and Clarke, 1984, 1986). Several minor peaks were also evident by the end of the ester hydrolysis (Fig. 4, asterisks). Importantly, only the isopeptide material was found to act as a substrate for erythrocyte carboxyl methyltransferase activity (Fig. 4).

When the hydrolysis experiment was performed at physiological temperature and pH (37 °C, pH 7.4), the chemical hydrolysis of tetragastrin methyl ester was slower than at pH 9.5, with a half-time of ester loss of 124 min (Fig. 5). Again, as at pH 9.5, the imide formed as an intermediate product. The imide then hydrolyzed with an apparent half-life of 114 min to a mixture of L-iso and normal peptides (Fig. 5, right). The imide structure was confirmed by recovering it in pure form and demonstrating its hydrolysis to iso and normal forms with alkaline treatment (data not shown).

By the end of the pH 7.4 ester hydrolysis (Fig. 5), 80% of the starting ester material was converted to L-iso-tetragastrin. The remaining 20% was recovered as normal tetragastrin. Fewer minor products were formed by pH 7.4 hydrolysis (Fig. 5) compared to the pH 9.5 condition (Fig. 4). It is possible that the pH 9.5 ammonium acetate buffer was reactive, and some of the minor peaks appearing at pH 9.5 are amidated or acylated versions of tetragastrin. In support of this possibility, the de-esterification procedure was also performed at pH 9.5 using a sodium borate buffer, and the minor products were eliminated (data not shown).

Characterization of L-iso-Tetragastrin—Following chemical esterification/de-esterification, the normal and L-iso forms of
tetragastrin and L-iso-tetragastrin were included as internal standards. The fragments of this cleavage were analyzed by HPLC as described under "Materials and Methods." Standards of L-Asp-L-PheNH₂, L-isoAsp-L-PheNH₂, and the uncleaved standards of normal tetragastrin and L-iso-tetragastrin were included as internal standards in several analyses, with their elution positions as shown here. The peak marked by the bold arrow was present in each cyanogen bromide reaction mixture, and its UV absorbance was consistent with the expected N-terminal cleavage fragment L-Trp-L-Hse, where Hse is homoserine. Upper trace, cyanogen bromide cleavage of 700 pmol of purified L-iso-tetragastrin. Middle trace, cleavage of 800 pmol of normal tetragastrin. Lower trace, cleavage of a mixture of 400 pmol of normal tetragastrin and 350 pmol of L-iso-tetragastrin.

![Diagram 6: Products of tetragastrin methyl ester hydrolysis analyzed after cyanogen bromide cleavage, L-iso-Tetragastrin and normal tetragastrin were purified following the hydrolysis of tetragastrin methyl ester and were then cleaved by cyanogen bromide treatment. The fragments of this cleavage were analyzed by HPLC as described under "Materials and Methods." Standards of L-Asp-L-PheNH₂, L-isoAsp-L-PheNH₂, and the uncleaved standards of normal tetragastrin and L-iso-tetragastrin were included as internal standards in several analyses, with their elution positions as shown here. The peak marked by the bold arrow was present in each cyanogen bromide reaction mixture, and its UV absorbance was consistent with the expected N-terminal cleavage fragment L-Trp-L-Hse, where Hse is homoserine. Upper trace, cyanogen bromide cleavage of 700 pmol of purified L-iso-tetragastrin. Middle trace, cleavage of 800 pmol of normal tetragastrin. Lower trace, cleavage of a mixture of 400 pmol of normal tetragastrin and 350 pmol of L-iso-tetragastrin.]

![Diagram 7: Stoichiometry of enzymatic carboxyl methylation of L-iso-tetragastrin. Purified L-iso-tetragastrin trifluoroacetate (350 pmol) was incubated with partially purified erythrocyte carboxyl methyltransferase (10 µg of protein) and 2800 pmol of S-adenosylmethionine. The final reaction volume (40 µl) also contained S-adenosylhomocysteine hydroxylase from rabbit erythrocytes (4 µl; 30 units/µl containing 11 mg/ml enzyme protein and 20 mg/ml bovine serum albumin, Sigma), adenosine deaminase from hog kidney (1 µl of an ammonium sulfate suspension; 2 units/µl, Sigma), and the reaction was buffered by 75 mM sodium citrate, 1.5 mM Na-EDTA, 3% glycerol, 1.5 mM β-mercaptoethanol, pH 6. After incubation (37°C) for 60 min, the reaction was quenched with 50 µl of 1 M acetic acid, and 15 µl of the quenched sample (equivalent to 58 pmol of the starting peptide) was analyzed by the standard HPLC conditions described under "Materials and Methods." Based on UV absorbance, 90% of the starting L-iso-tetragastrin was enzymatically converted to the methylated product, L-iso-tetragastrin methyl ester.]]

**Table II**

| Peptide          | Trp | Met | Asp | Phe |
|------------------|-----|-----|-----|-----|
| Tetragastrin     | 1.00| 0.80| 0.60| 0.63|
| L-iso-Tetragastrin| 1.00| 0.43| 0.001| 0.003|

![Fig. 11507: Peptide Substrates for Protein Carboxyl Methyltransferase]

Leucine aminopeptidase digestion of L-iso- and normal tetragastrin Chromatographically purified tetragastrin (130 pmol) and L-iso-tetragastrin (140 pmol) from the pH 9.5 hydrolysis of tetragastrin methyl ester were digested (37°C) in 40-µl volumes containing 44 µg of hog kidney leucine aminopeptidase (Sigma; 100 units/mg) and buffered by 0.05 M Tris-HCl, 2.5 mM MgCl₂, pH 8, for 19 h. Ratios of released amino acids were then quantified by the orthophthalaldehyde derivatization method described under "Materials and Methods."
was chromatographically identical to the synthetic product.

Studies with Other Peptides—Several other aspartyl peptides were chemically esterified and de-esterified at pH 7.4 and were then tested as substrates for erythrocyte carboxyl methyltransferase. The first four peptides listed in Table III were not detectably methylated by the activity until they underwent chemical treatment. Each of these peptides contains an aspartyl residue in a unique sequence and at a variable distance from the amino and carboxyl ends. Peptide 4 (Table III) also contains an asparagine residue which may possibly deamidate and rearrange spontaneously (Johnson et al., 1985; Clarke, 1985), although it is not known if the present chemical conditions would promote the deamidation of this peptide. The precise structures of the methyl acceptor species obtained from peptides 1 through 4 by chemical esterification/de-esterification treatment have not been determined, but by analogy to the results with tetragastrin it is expected that L-iso forms of the peptides are chemically generated. Unlike the situation with the C-terminally amidated tetragastrin, peptides 1 and 2 (Table III) have a free C terminus that would be esterified during the chemical procedure. We have not assayed for C-terminal methyl esters or for the loss of C-terminal esters in the de-esterification step.

As expected, the last two peptides in Table III (peptides 5 and 6) were not enzymatic substrates either before or after chemical treatment. Neither peptide contains an asparyl group, but each does contain a glutamic acid site. Esters of glutamyl sites in polypeptides are relatively stable groups at mildly alkaline pH (Terwilliger and Clarke, 1981; Kleene et al., 1977) and are probably not easily subject to intramolecular displacement with subsequent glutarimide formation and bond rearrangements. It appears that an asparyl residue is necessary for the generation of new methyltransferase substrates by this method.

DISCUSSION

We have developed a general method to convert normal L-aspartyl residues to β-isomerized L-aspartyl residues (Fig. 1).

In this study, we have used a two-step procedure to obtain L-iso-tetragastrin from normal tetragastrin in 50% yield. This L-isoaspartyl form of tetragastrin is recognized and methylated by erythrocyte protein methyltransferase activity, whereas the normal form is not. These experiments thus demonstrate that normal aspartyl peptides can be rearranged by simple chemical treatments, and this chemical method can be usefully applied to generate new methyltransferase substrates. A parallel chemical method may also be useful to convert isopeptides back to normal aspartyl peptides, although the yield of this reaction may be low since isopeptide formation is generally favored (Bernhard, 1983; Johnson and Asawd, 1985; Murray and Clarke, 1986).

Using this methodology, we have extended the known in vitro range of substrate specificity of erythrocyte protein carboxyl methyltransferase. We find that a wide range of aspartyl peptides can be converted into methyltransferase substrates. For one peptide, L-iso-tetragastrin, the precise structure of the new methyl acceptor species has been determined. The other newly generated substrates (Table III) may also be L-isoaspartyl peptides, although other structures should be considered. It is possible that the chemical methods developed here can result in the epimerization of normal L-aspartate residues to yield D-aspartyl derivatives which may act as enzymatic substrates. In fact, preliminary work with longer reaction times and higher temperatures during the de-esterification of tetragastrin methyl ester at pH 9.5 has resulted in product peptides with large D-aspartic acid content. Such D-aspartic acid derivatives of tetragastrin have not yet been found to be enzymatically methylated, but other D-aspartic acid-containing peptides might be expected to be

| Peptide | Treatment | Concentration | Enzymatic methylation (μM) |
|---------|-----------|--------------|---------------------------|
| Phe-Asp-Ala-Ser-Val | Untreated | 400 | 7.5 ± 0.8 |
| Arg-Lys-Asp-Val-Tyr | Untreated | 400 | 39.2 ± 1.7 |
| <Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH₂ | Untreated | 88 | 14.3 ± 3.8 |
| <Glu-Ala-Asp-Pro-Lys-Phe-Gly-Leu-Met-NH₂ | Untreated | 44 | 34.1 ± 0.8 |
| Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly | Untreated | 2.6 | 0.1 ± 1.3 |
| Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg | Untreated | 1.3 | 94.1 ± 3.5 |

DISCUSSION

We have developed a general method to convert normal L-aspartyl residues to β-isomerized L-aspartyl residues (Fig. 1).
**Peptide Substrates for Protein Carboxyl Methyltransferase**

**TABLE IV**

Enzymatic carboxyl methylation of L-isoaspartyl peptides and derivatives

The peptides shown were each tested as enzymatic substrates using preparations of human erythrocyte protein carboxyl methyltransferase. Assays were performed at pH 7.0 except in the case of L-iso-tetragastrin which was tested at pH 7.4.

| Compound                | R₁ | R₂ | Methyl acceptor | Vₓₓ * | References |
|-------------------------|----|----|-----------------|-------|------------|
| Gly-L-Asn               | Gly| H  | >5 mM*          |       | This study |
| N-Acetyl-L-Asn          | O  | H  | >190 mM*        |       | This study |
| L-Asp-β-Gly             | H  | Gly| >7 mM*          |       | This study |
| L-iso-Tetragastrin      | Trp-Met | Phe-NH₂ | 5 µM | 0.45 | This study |
| ACTH-related isopeptide | Val-Tyr-Pro | Gly-Ala | 6 µM | 1.3 | Murray and Clarke, 1984 |
| L-iso-Nonapeptide       | Lys-Ala-Ser-Ala | Leu-Ala-Lys-Tyr | 0.4 µM | 0.96 |          |

* Value given relative to that observed with saturating concentration of ovalbumin as a methyl acceptor.
* At the indicated concentration of substrate, methylation was not detected.

Little is now known of the amino acid sequences surrounding methyl acceptor sites in *vivo*. Whether the sites defined here and in other *in vitro* studies resemble *in vivo* sites will not be known until a methylated site in a cellular protein is fully examined. Our *in vitro* work indicates that erythrocyte protein carboxyl methyltransferase has broad sequence specificity that may enable it to recognize many altered protein sequences. An important question now is which sequences are most likely to become altered in cellular proteins. The likelihood of rearrangement is presumably dependent on many structural and environmental factors; it is of interest to note that succinimides are likely intermediates in the generation of cellular L-isoaspartyl and D-aspartyl residues from protein L-aspartyl and L-asparaginyl residues (Johnson and Aswad, 1985; Aswad, 1984a; Johnson et al., 1985; Murray and Clarke, 1984).

REFERENCES

Aswad, D. W. (1984a) *J. Biol. Chem.* 259, 19714-10721
Aswad, D. W. (1984b) *Anal. Biochem.* 137, 405-409
Barber, J. R., and Clarke, S. (1985) *Biochemistry* 24, 4867-4871
Bernhard, S. A. (1983) *Ann. N. Y. Acad. Sci.* 421, 28-40
Boyd, A., Kendall, K., and Simon, M. I. (1983) *Nature* 301, 625-626
Clarke, S. (1985) *Annu. Rev. Biochem.* 54, 479-506
Freitag, C., and Clarke, S. (1981) *J. Biol. Chem.* 256, 6102-6108
Gosselin, M. L., and Liss, M. (1985) *J. Protein Chem.* 4, 129-132
Johnson, B. A., and Aswad, D. W. (1985) *Biochemistry* 24, 2581-2586
Johnson, B. A., Freitag, N. E., and Aswad, D. W. (1985) *J. Biol. Chem.* 260, 10913-10916
Jones, B. N., Paabo, S., and Stein, S. (1981) *J. Liq. Chromatogr.* 4, 565-568
Kehry, M. R., Bond, M. W., Hunkapiller, M. W., and Dshquist, F. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 3928-3922
Kim, S. (1974) *Arch. Biochem. Biophys.* 161, 652-657
Kim, S., Choi, J., and Jun, G. J. (1983) *J. Biochem. Biophys. Methods* 8, 9-14
Kleene, S. J., Toews, M. L., and Adler, J. (1977) *J. Biol. Chem.* 252, 3214-3218
Macfarlane, D. E. (1984) *J. Biol. Chem.* 259, 1357-1362
McFadden, P. N., and Clarke, S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 79, 2460-2464
Murray, E. D., Jr., and Clarke, S. (1984) *J. Biol. Chem.* 259, 10722-10732
Murray, E. D., Jr., and Clarke, S. (1986) *J. Biol. Chem.* 261, 301-316
O'Connor, C. M., and Clarke, S. (1983) *J. Biol. Chem.* 258, 5485-5492
O'Connor, C. M., and Clarke, S. (1984) *J. Biol. Chem.* 259, 2570-2577
O'Connor, C. M., Aswad, D. W., and Clarke, S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 81, 7757-7761
Paik, W. K., and Kim, S. (1980) *Protein Methylation*, pp. 202-230, John Wiley and Sons, New York
Peptide Substrates for Protein Carboxyl Methyltransferase

Philip N. McFadden and Steven Clarke

MATERIALS AND METHODS

Peptides. Residues are in the L-configuration unless indicated otherwise. Tetragastrin-HCl (Try-Asp-Arg-Phe-Glu) from both Sigma and Vega was used. The following peptides were from Sigma: Arg-Asp-His; Ser-Tyr-Arg-Glu-His-Phe-Tyr-Arg; Glu-Ala-Glu-Ala-Glu-His-Leu-Met-His-Arg; (physalolin) Phe-Ser-Tyr-Gly-Ala-Glu-Glu-Gly-His-Glu-Ile-Pro-Glu-Glu-Glu. All of the above were purified, typically on a 0.5 mg % scale by dissolving them in solvent A and injecting 200 ul volumes onto the HPLC system described above. Elution over desiccant in the dark at -20°C was performed under laboratory atmosphere and under laboratory atmosphere. The scale and duration of each de-esterification reaction are described in the Figure Legends. In the case of the volatile ammonium acetate buffer salts, peptides could be isolated by freezing and lyophilizing the reaction mixture.

Protein Carboxyl methyltransferase

Peptide Characterization

Amino Acid Analysis. Tetragastrin peptides were acid hydrolyzed with 6 M HCl solution at 115°C for 18 hours in vacuum. Tetragastrin peptides were enzymatically hydrolyzed by Weissman and Clarke, 1984. Methyl esterification. Peptide salts were esterified with reagent grade methyl Esterification. Peptide salts were esterified with reagent grade methyl transferase away from the bulk of the hemoglobin (Kim et al., 1983). Methyl Esterification. Peptide salts were esterified with reagent grade methyl transferase away from the bulk of the hemoglobin (Kim et al., 1983). Methyl Esterification. Peptide salts were esterified with reagent grade methyl transferase away from the bulk of the hemoglobin (Kim et al., 1983). Methyl Esterification. Peptide salts were esterified with reagent grade methyl transferase away from the bulk of the hemoglobin (Kim et al., 1983).

Enzyme Purification. All steps were performed on ice or in a 4°C cold room. Solid ammonium sulfate was added to stirred cytosol to 55% saturation (11.6 g per 100 ml) to precipitate protein carboxyl methyltransferase from the bulk of the hemoglobin (Kim et al., 1983). The material in the 55% ammonium sulfate pellet was dissolved in Buffer C above and dialyzed overnight against 25 volumes of Buffer C (350 mM potassium phosphate buffer, pH 7.4) as described below. Separate methyltransferase assays were performed using either ovalbumin (40 mg/ml) or esterified/de-esterified tetragastrin as methyl acceptor substrates. The peak fractions of carboxyl methyltransferase activity were pooled, and this solution was concentrated approximately 10-fold by ammosia by transferring it to a 3500 MW cutoff dialysis bag and packing the outside of the bag for several hours with dry Sephadex G-200 gel.

Vapor Diffusion Assay for Methyltransferase and Methyl-Acceptor Substrates. This assay method is based on the determination of radioactive methyl transferred from enzyme-derived methyl esters to isolated substrates. The general procedure of Macfarlane (1984), N-Acyl-N-methylthionine-259 (Boyce and Clarke, 1981) was mixed with N-Acyl-N-methylthionine-259 (Boyce and Clarke, 1981) was mixed with N-Acyl-N-methylthionine-259 (Boyce and Clarke, 1981) was mixed with N-Acyl-N-methylthionine-259 (Boyce and Clarke, 1981) was mixed with N-Acyl-N-methylthionine-259 (Boyce and Clarke, 1981) was mixed with N-Acyl-N-methylthionine-259 (Boyce and Clarke, 1981). The material in the 55% ammonium sulfate pellet was dissolved in Buffer C above and dialyzed overnight against 25 volumes of Buffer C (350 mM potassium phosphate buffer, pH 7.4) as described below. Separate methyltransferase assays were performed using either ovalbumin (40 mg/ml) or esterified/de-esterified tetragastrin as methyl acceptor substrates. The peak fractions of carboxyl methyltransferase activity were pooled, and this solution was concentrated approximately 10-fold by ammosia by transferring it to a 3500 MW cutoff dialysis bag and packing the outside of the bag for several hours with dry Sephadex G-200 gel.
phenylisothiocyanate and analyzed at 254 nm using the Waters Pico-Tag protocol.

D/L Aspartic Acid Analysis. Tetragastrin peptides were acid hydrolyzed as above and the residue was reacted with an optically active and fluorogenic reagent previously described (Aswad, 1984b). The resulting diastereomeric adducts of D and L-aspartic acid with N-acetyl-L-cysteine and orthophthalaldehyde were separated and the D- to L- aspartic acid ratio determined as described by Aswad (1984b) and modified by Murray and Clarke (1984).

Cyanogen bromide cleavage reaction. The cyanogen bromide cleavage of tetragastrin peptides was performed with one-volume of the peptide (dissolved in water) and one-volume of reagent (0.01 g/ml cyanogen bromide, J.T. Baker, in 88% formic acid. Mallinckrodt) in polyethylene tubes incubated in a 50°C water bath. After one hour, the reactions were frozen and lyophilized, and the residue was resuspended in water for analysis by HPLC. Samples were injected onto the Waters/Alltech System described in the HPLC section above, and elution was by a linear gradient of 0% to 40% solvent B over 40 minutes.

Synthesis of standard L-iso-Asp-Phe-NH₂. This expected product of the cyanogen bromide cleavage of L-iso-tetragastrin was synthesized by coupling N-carbobenzoxy-L-aspartic acid alpha-methyl ester (1 mol, Vega) to L-phenylalanine amide (1 mol, Vega) in tetrahydrofuran (6 ml) using dicyclohexylcarbodiimide (1.1 mmol) as coupling agent with 2 mmol 1-hydroxybenzotriazole and 1.2 mmol triethylamine also present. After removal of the solid dicyclohexylurea by filtration, the solvent was removed by vacuum and neutral peptide material was recovered after solvent extractions with ethyl acetate, aqueous sodium bicarbonate and aqueous citric acid. The neutral material (25% yield) was deprotected by one hour treatment with palladium catalyst on a polymeric support (Pierce Palladium-PET beads) suspended in 1:4 formic acid (88%)/tetrahydrofuran. A single UV214 absorbing product was evident upon purification by preparative reversed phase HPLC (above) where the product eluted slightly before a standard of normal L-Asp-L-Phe-NH₂ (Sigma). The product was judged to be L-iso-Asp-L-Phe-NH₂ by its failure to be cleaved by leucine aminopeptidase (the normal standard was cleaved) and by its amino acid composition (1 mole L-Asp, 1 mole Phe).