Metabolism of a Synthetic L-Isoaspartyl-containing Hexapeptide in Erythrocyte Extracts

ENZYMATIC METHYL ESTERIFICATION IS FOLLOWED BY NONENZYMATIC SUCCINIMIDE FORMATION*

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E. David Murray, Jr.§ and Steven Clarke§
From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024

The synthetic peptide, L-Val-L-Tyr-L-Pro-L-isoAsp-Gly-L-Ala, is a substrate for the erythrocyte and brain protein carboxyl methyltransferases. These enzymes catalyze the methyl esterification of the free α-carboxyl group of the isoaspartyl residue, to which the glycol residue is linked through the side chain β-carboxyl group. In this work, we show that the α-methyl ester of this peptide is rapidly demethylated (t½ = 4 min at 37 °C, pH 7.4) in erythrocyte cytosolic extracts and that the product of this reaction appears to be the succinimide ring derivative of the peptide. The rate of demethylation, measured at either pH 6.0 or 7.4, was the same in buffer and erythrocyte extracts, suggesting that succinimide formation was a nonenzymatic reaction. The L-succinimide is more stable than the ester, but can be hydrolyzed in buffer at pH 7.4 (t½ = 180 min at 37 °C) to give a mixture of about 75% isoaspartyl peptide and 25% normal aspartyl peptide. The metabolism of the succinimide hexapeptide in erythrocyte extracts appears to be more complex, however. The implications of this work for the methylation and demethylation of cellular proteins containing structurally altered aspartyl residues are discussed.

Protein carboxyl methyltransferases from human erythrocytes and bovine brain catalyze the methylation of abnormal aspartyl residues in cellular proteins and synthetic peptides (1–2). These sites of methylation appear to include the β-carboxyl group of racemized residues, in which the terminal α-carbon is in the D-configuration (3) and the α-carboxyl group of isomerized L-aspartyl residues in which the peptide chain is linked via the side chain β-carboxyl group (4–5). The function of these methyltransferases is not known, but it has been proposed that these enzymes play a surveillance role in the cell to identify abnormal proteins and may participate in their degradation or rejuvenation.

The development of a synthetic peptide substrate for eukaryotic protein carboxyl methyltransferases has provided an approach to understanding the function of these enzymes. This peptide substrate, L-Val-L-Tyr-L-Pro-L-isoAsp-Gly-L-Ala, is stoichiometrically methylated at the free α-carboxyl group of the isoaspartic acid residue by both the red blood cell and brain methyltransferases (5). Substrates previously identified for these enzymes were proteins and peptides in which only a small subpopulation of the molecules contained altered aspartyl residues capable of acting as substrates (6–11). It was not possible to investigate the metabolism of these methyl esterified proteins, labeled with a radioactive methyl group, because the subpopulation of molecules which contains D-aspartyl and/or L-isoaspartyl residues becomes lost among the much larger population of unmodified proteins following the demethylation reaction. However, the demethylation and subsequent metabolism of stoichiometric methyl-accepting peptides can be tested.

As a first step in our analysis, the fate of the methyl ester of the L-isohexapeptide characterized previously (5) has been examined following the methylation step. We wanted to determine whether there is a methylesterase activity present in erythrocyte cytosol and to characterize the product(s) of the initial demethylation reaction both in cell extracts and in buffer. A methylesterase activity, active at acidic pH on gelatin methyl esters formed by the erythrocyte protein carboxyl methyltransferase, has been detected and characterized in kidney extracts (12–14). It is unclear, however, whether this activity is present in other cell types and whether it has a specific role in the demethylation of cellular protein methyl esters. The results presented in this paper suggest that the methyl ester of the L-isohexapeptide is metabolized in erythrocyte extracts in a nonenzymatic reaction leading to the formation of the peptide containing an intramolecular succinimide ring.

EXPERIMENTAL PROCEDURES

Synthetic Peptides

L-Val-L-Tyr-L-Pro-L-isoAsp-Gly-L-Ala and the corresponding L-succinimide were synthesized, purified, and characterized as described previously (5). The concentration of the peptides used in these experiments was determined using a UV extinction coefficient of 1402 M⁻¹ cm⁻¹ at 274 nm.

High Performance Liquid Chromatography

The system for liquid chromatography consisted of two Waters M-45 pumps, an M660 solvent programmer, a U6K injector, and a model 441 absorbance detector (214 nm). The reverse phase C₁₈ columns (4.6 × 250 mm, 5-μm spherical resin) were Econosphere columns from Alltech. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.1% trifluoroacetic acid in 10% water:90% acetonitrile.
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These solvents were prepared using a 10% stock of Pierce Sequential grade trifluoroacetic acid as described previously (5). The column was equilibrated at room temperature in solvent A. Samples were eluted at 1 ml/min by a linear gradient of 0-40% solvent B in 40 min. This system can resolve the related hexapeptides containing L-isoAsp residues (25-min elution time), L-Asp residues (26.5 min), L-succinimide residues (29 min), and L-isoAsp $\alpha$-methyl ester residues (30 min) (see below).

**Time Course of $\alpha$-Hexapeptide Imide Opening**

The rate of ring opening of the $\alpha$-hexapeptide imide in buffer was investigated at two pH values. At pH 6, 130 $\mu$l of 1.9 mM hexapeptide imide in H$_2$O was mixed with 370 $\mu$l of 0.2 mM sodium succinate, pH 6.0 (the final pH was measured at 6.0), and incubated at 37°C. At various time intervals, aliquots (50 $\mu$l) were withdrawn and added to 50 $\mu$l of a quench solution composed of 4% trifluoroacetic acid. For the pH 7.4 incubation, 400 $\mu$l of 0.28 mM hexapeptide imide in H$_2$O was mixed with 400 $\mu$l of 0.2 mM sodium HEPES,$^3$ pH 7.4 (final pH was 7.4), and placed in a 37°C bath. At various times, 40-$\mu$l aliquots were withdrawn and quenched as described above. The amount of hexapeptide imide remaining was analyzed in each case by HPLC as described above.

**Preparation of Erythrocyte Cytosol as Source of Carboxyl Methyltransferase**

Erythrocyte cytosol at a protein concentration of about 60 mg/ml was prepared from washed cells by lysis in 4 volumes of 4.5 mM sodium phosphate, 4.5 mM disodium EDTA, 13.5 mM B-mercaptoethanol, and 10% glycerol at pH 8.0 as described (5).

**Vapor Diffusion Assays for $[^3H]$Methanol**

Procedure A—Sample (80 $\mu$l) was mixed with an equal volume of 0.6 M sodium borate, 1% sodium dodecyl sulfate, pH 10.2, in a 400-$\mu$l polycarbonate microfuge tube. This tube was placed uncoated in a larger vial containing 2.4 ml of liquid scintillation mixture as described (5). The amount of $[^3H]$methanol in each sample was determined from the radioactivity which was found in the liquid scintillation fluid after 24 h at room temperature, after a correction for the efficiency of methanol transfer (about 30%) was made (5).

Procedure B (Filter Paper Assay)—A new variation on the methanol vapor diffusion assay for protein carboxyl methyltransferase activity was recently suggested by Macfarlane (15). Briefly, a base-quenched sample containing protein carboxyl methyl esters was applied to a piece of thick filter paper which was then placed in the neck of a capped scintillation vial containing scintillation fluid. The vial was left undisturbed for several hours at room temperature to allow the radiolabeled methanol to diffuse from the filter paper into the scintillation fluid. After the filter paper was removed, the radioactivity in the vial was determined. Specifically, an aliquot (10 $\mu$l) of sample, which was mixed with 38 $\mu$l of 0.2 M NaOH, 1% sodium dodecyl sulfate, and then immediately applied to a 8.8-cm strip of accordian-pleat folded filter paper backing for slab gels (Bio-Rad catalog no. 165-0921). The paper was then inserted into the neck of a 22-ml plastic scintillation vial containing 10 ml of ACS II counting fluid (Amersham Corp.). The vial was capped, and after 2 h the folded strip of paper was removed and the vial recapped and counted. Control experiments with $[^3H]$methanol and various buffers showed that for a volume of 40 $\mu$l spotted on the paper, transfer of $[^3H]$methanol was essentially complete (100% yield) after 2 h.

**Incubation of Peptides in Erythrocyte Cytosol**

Hexapeptides were incubated at concentrations of 10 $\mu$m at 37°C with erythrocyte cytosol (19 mg/ml protein) containing 120 mM sodium HEPES buffer, pH 7.4. At various time intervals, 100-$\mu$l aliquots were removed and added to an equal volume of 10% trifluoroacetic acid and denatured proteins removed by centrifugation as described in the legend to Fig. 3. The amount of the normal, iso-, and succinimide forms of the peptides were determined by HPLC as described previously (5).

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AdoMet, S-adenosyl-L-methionine; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

**RESULTS**

**Formation and Turnover of L-Isohexapeptide Methyl Ester in Erythrocyte Cytosol—Incubation of L-Val-L-Tyr-L-Pro-L-isoAsp-Gly-L-Ala with $[^3H]$AdoMet and erythrocyte cytosol leads to the enzymatically catalyzed methylation of this peptide (5). In this previous study, the stability of the peptide methyl ester in the erythrocyte cytosol incubation mixture was not determined. To investigate the possibility that a rapid demethylation reaction might follow the methyl esterification reaction under these conditions, we developed an assay that would distinguish between $[^3H]$methyl esters and the product of demethylation, free $[^3H]$methanol. This was done by assaying base-labile, volatile radioactivity in aliquots of the incubation mixture with and without prior lyophilization to remove free $[^3H]$methanol. Radioactivity detected in samples which were not lyophilized would thus represent $[^3H]$methanol as well as $[^3H]$methyl esters, whereas that from lyophilized samples would only represent $[^3H]$methyl esters.

The results of such experiments, performed where the incubation was carried out at either pH 6.0 or at the physiological pH value of 7.4, are shown in Fig. 1. At pH 6.0, there appears to be little turnover of methyl esters for the first 30 min of the incubation because the total radioactivity (free $[^3H]$methyl alcohol + $[^3H]$methyl esters) was essentially equal to the radioactivity present in $[^3H]$methyl esters alone. However, at incubation times of 60 min or greater, a substantial fraction of the radioactivity was present as free $[^3H]$methanol and indicates that demethylation does occur under these conditions. At pH 7.4, the turnover of methyl esters appears to be much more rapid, and the bulk of the radioactivity in the incubation mixtures was present as $[^3H]$methanol, even at the early time points. Although the total rate of methyl esterification was about the same at pH 6.0 and pH 7.4, the methyl esters formed at pH 7.4 are much more rapidly demethylated to form $[^3H]$methanol. In all of these experiments, control reactions were performed in the absence of the isohexapeptide substrate; a small background resulting from $[^3H]$AdoMet decomposition and the methylation of endogenous cytosolic proteins has been subtracted to obtain the results shown in Fig. 1.

From these data, it was possible to calculate the rate of demethylation of the L-isohexapeptide methyl ester at both pH values. The reaction pathway is shown in Fig. 2. The rate equation for the demethylation reaction can be expressed as follows:

$$\frac{d[^3H]CH_3OH}{dt} = k_2 \text{[peptide ester]}$$

For the data in Fig. 1, this equation can be approximated by

$$\Delta[^3H]CH_3OH = k_2 \frac{[^3H]CH_3OH}{\Delta t} \text{[peptide ester]}$$

and rearranged to give

$$k_2 = \frac{\Delta[^3H]CH_3OH}{\Delta t \text{[peptide ester]}}$$

Since the values for peptide ester concentration were measured as a function of time in Fig. 1 and since the free methanol was equal to the total methanol minus the peptide esters, the data in Fig. 1 can be used to determine a value for $k_2$. The amount of free methanol for any time point can be determined by subtracting the amount of peptide methyl ester (determined from the lyophilized sample) from the total amount of methanol and methyl ester present (determined from the
Demethylation of Purified L-Isohexapeptide Methyl Ester in Buffer Occurs at the Same Rate as Demethylation in Erythrocyte Extracts—Radiolabeled L-isohexapeptide methyl ester can be purified from an erythrocyte cytosol methylation mixture by HPLC (Fig. 3). Incubation of the purified L-isohexapeptide methyl ester in different pH buffers would give a direct measure of $k_2$ in the absence of any erythrocyte enzymes. A comparison of values, thus obtained, with those from previous demethylation experiments performed in the presence of erythrocyte cytosol would provide information as to whether a methylesterase activity exists in the erythrocyte cytosol. In the experiment shown in Fig. 4, the half-times of demethylation of the L-isohexapeptide methyl ester in buffers of pH 6.0 and 7.4 were 39.7 and 4.3 min, respectively. These times are essentially identical to those determined by other methods in erythrocyte cytosol (Table I, see above). The rate of demethylation in buffer is very similar to that in cytosol at the same pH, thus providing no evidence for the existence of a methylesterase activity.

**A Succinimide Is the Product of the Demethylation Reaction**—The rapid rate of demethylation of the peptide methyl ester was not consistent with a direct hydrolysis mechanism in which water or hydroxyl ion directly attacks the carbonyl carbon of the ester (18, 22). On the other hand, succinimide formation (see Fig. 2) has been observed with other types of peptide esters (16, 19), and the hexapeptide succinimide might be the immediate demethylation product of the hexapeptide methyl ester in this study. Since we have previously characterized the HPLC elution position of the succinimide form of this peptide (5), we performed the experiment shown in Fig. 5 to separate the products of the methylation and demethylation reactions. Here, the isohexapeptide was incubated with erythrocyte cytosol and nons isotopically labeled AdoMet. The composition of the incubation mixture was characterized at various times by HPLC, which provides excellent separation of the hexapeptide methyl ester, succinimide, and iso- and normal hexapeptide forms. The data in Figs. 5 and 6 show an initial conversion of the L-isohexapeptide to the methyl ester followed by conversion to a product which elutes in the expected position of the iso- and normal hexapeptide forms. The ratio of absorbance in these peaks of 3.6:1 (isopeptide: normal peptide) was similar to the value of 3.0:1 obtained when the purified hexapeptide succinimide was hydrolyzed (see below).

It was possible to measure the rate of demethylation of the L-isohexapeptide α-methyl ester at pH 6.0 by measuring the rate of succinimide formation. The value obtained for $k_2$ from the data in Figs. 5 and 6 was $1.66 \pm 0.53 \text{ S.D.)} \times 10^{-2} \text{ min}^{-1}$, giving an average value for the $t_{1/2}$ of 41.8 min. This latter value was very similar to that calculated from the measurement of $[\text{H}]$methanol production in Fig. 1 (Table I).

Formation of the hexapeptide imide upon loss of the methyl ester group indicates that the methylation does, indeed, occur on the α-carboxyl group of the isoaspartyl residue, rather than on the C-terminal carboxyl group, since no reasonable mechanism can be proposed for formation of the hexapeptide imide upon demethylation of the C-terminal carboxyl group.

Hydrolysis of the Hexapeptide Succinimide—To compare the rate of ring opening of the L-hexapeptide imide to the demethylation rate of the peptide ester, the hydrolysis of the succinimide to the normal and isopeptide in buffer at pH 6
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FIG. 2. Pathways of enzymatic and nonenzymatic metabolism of the L-isohexapeptide in erythrocyte cytosol.

| pH | Reaction | Medium | $t_{1/2}$ |
|----|----------|--------|----------|
| 6.0 | Demethylation | Cytosol | 39.4$^a$ |
|     |           | Buffer | 41.8$^a$ |
|     | Succinimide ring hydrolysis | Buffer | 1660$^d$ |
| 7.4 | Demethylation | Cytosol | 3.6$^a$ |
|     |           | Buffer | 4.3$^a$ |
|     | Succinimide ring hydrolysis | Buffer | 180$^d$ |

$^a$ Determined from the rate of $[^3H]$methanol production from the data in Fig. 1.

$^b$ Determined from the rate of succinimide formation from the data in Figs. 5 and 6.

$^c$ Determined by the rate of $[^3H]$methanol production (Macfarlane assay, Procedure B) from the data in Fig. 4.

$^d$ Determined by the production of L-isooaspartyl and L-aspartyl hexapeptides from the L-succinimide as described under "Experimental Procedures."

and pH 7.4 was measured. The relative concentrations of these products were measured by the area of their absorbance peak on HPLC. At pH 6.0, the imide opens with a half-time of 1660 min and at pH 7.4 with a half-time of 180 min (Table I). At both pH values, the products consist of 75% isopeptide and 25% normal peptide. We are presently characterizing the products of succinimide metabolism in red cell cytosolic extracts at pH 7.4. Preliminary results indicate that the loss of L-succinimide in cytosolic extracts at pH 7.4 and 37 °C can be faster than in buffer at the same pH. Because we have not detected the formation of the normal or the isohexapeptide in the HPLC assay under these conditions (see "Experimental Procedures"), it is possible that the rapid loss may be due to proteolysis in addition to the ring-opening reaction.

Determination of the Methylation Stoichiometry of Synthetic Peptides—It was previously shown that the L-isohexapeptide was capable of forming about one methyl ester per peptide (5). The total $[^3H]$methanol measured in the present experiment with 1 μM peptide at pH 6.0 shows that approximately 40% of the L-isohexapeptide was methylated (Fig. 1, top panel), which is similar to the 80% methylation achieved with a concentration of 0.25 μM peptide. The results of these assays, which measure both free methanol and peptide methyl
esterases, probably reflect the total number of esters formed during the incubation period. There was no detectable formation of methyl esters when cytosol was incubated with S-adenosylmethionine in the absence of isohexapeptide. Additionally, the remethylation of previously methylated and demethylated peptides did not appear to contribute to the overall methylation stoichiometry. The succinimide product of the demethylation reaction was not a substrate for the methyltransferase (5), and the amount of L-isohexapeptide formed from imide hydrolysis was insignificant in these experiments, since the rate of imide opening was approximately 40 times slower than the demethylation rate (see below, Table I).

**DISCUSSION**

**Rapid Demethylation of L-Val-L-Tyr-L-Pro-L-isoAsp-a-Methyl Ester-Gly-L-Ala Is Apparently a Nonenzymatic Process in Erythrocyte Cell Extracts**—A specific enzyme was found in bacterial cells that catalyzes the demethylation of chemoreceptor protein methyl esters (20, 21), and a similar activity was found in rat kidney cells that hydrolyzes gelatin methyl esters formed by the erythrocyte methyltransferase (12-14). If a demethylase existed in erythrocyte cytosol that catalyzed the reversal of the methyl esterification reaction in these cells, then the rate of demethylation of the L-isohexapeptide a-methyl ester hexapeptide might be expected to be greater in the presence of the cytosolic fraction than it was in buffer at the same pH. Table I shows that the rate of demethylation for the hexapeptide was the same in the presence and absence of erythrocyte cytosol at either pH 6.0 or 7.4. Our failure to detect erythrocyte isopeptide esterase activity is consistent with the previous failure to detect an erythrocyte methyl esterase that recognizes the physiological membrane protein substrates of the methyltransferase (22). In this latter study, isolated a-H-methylated erythrocyte membranes labeled by incubation of intact erythrocytes with L-[methyl-3H]methionine were incubated in the presence and absence of added erythrocyte lysate. No increase in the rate of demethylation in the presence of the lysate fraction was found (22).

**Peptide Succinimide as the Intermediate of Ester Hydrolysis**—The spontaneous rates of demethylation of the esters of many methylated erythrocyte proteins were comparable to that of the synthetic peptide ester studied here and were thus rapid on a physiological time scale (Table II, Refs. 18, 19, 22). The rapid spontaneous demethylation seems likely to result from demethylation mechanisms that involve the intermediate formation of intramolecular 5-membered rings. These derivatives include peptide succinimides (16-19, 22), anhydrides (22), or 5(4H)-oxazolones (23). In the present case, it is likely that facile formation of the succinimide product (Figs. 2 and 5) can account for the demethylation rate. A succinimide intermediate also appears to be the demethylation product of an a-methyl ester of deamidated adrenocorticotropic hormone (19). Although the formation of these intermediates has not been directly demonstrated from erythrocyte protein methyl esters, the rates of protein demethylation were consistent with intermediate formation (16-18, 22). It should be noted, however, that the rate of demethylation of at least one class of erythrocyte membrane protein methyl esters was about 375 times slower than the rate of the synthetic peptide methyl ester (Table II). Perhaps three-dimensional restrictions in protein conformation, or the presence of bulky residues nearby the aspartyl methyl ester, can slow the demethylation rate or restrict the utilization of different chemical demethylation pathways.

On the other hand, the spontaneous demethylation rate of

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**FIG. 3.** HPLC purification of isotope-labeled L-isohexapeptide methyl ester. The synthetic isopeptide was enzymatically methylylated in a mixture consisting of 20 μM of S-adenosyl-[methyl-3H]methionine (Amersham Corp., 15 Ci/mmol, 66.7 μM), 40 μM of 0.2 M sodium citrate, pH 5.0, 10 μM of 100 μM L-isohexapeptide, and 30 μM of erythrocyte cytosol as a source of methyltransferase (70.4 mg of protein/ml). The mixture was incubated for 45 min at 37°C before being quenched with 100 μM of 10% trifluoroacetic acid. The denatured proteins was removed by centrifugation for 30 min in a Beckman microfuge B. The methylated peptide was separated from the other incubation components by HPLC as described under "Experimental Procedures." Fractions (1 min) were collected and assayed for base-labile radioactivity, 10 μl of each fraction was added to 1 ml of ACS II liquid scintillation fluid and counted in a Packard model 3255 Liquid Scintillation Spectrometer. The absorbance of the effluent at 214 nm is shown by a continuous line. The radioactivity for each fraction is indicated by the bar graph.

**FIG. 4.** Demethylation of purified, radiolabeled L-isohexapeptide ester. Lyophilized HPLC-purified radiolabeled L-isohexapeptide ester was resuspended in water to a radioactive concentration of approximately 65 cpm/μl. Equal volumes of the peptide ester and buffer (either 0.2 M sodium succinate, pH 6.0, or 0.2 M sodium HEPES, pH 7.4) were mixed and incubated at 37°C. At various times, two 10-μl aliquots were withdrawn and mixed with 30 μl of either 0.6 M sodium phosphate, 1% sodium dodecyl sulfate, pH 1.0, or 0.2 M NaOH, 1% sodium dodecyl sulfate. This mixture was then applied to accordian-pleat folded filter paper strips, and the assay for volatilization of [3H]methanol was carried out as described under "Experimental Procedures" (Procedure B). Here, since the peptide ester was base-labile, the base quench was a measure of the total radioactivity as methyl esters and methanol present in a sample at any time, while the acid quench will provide a measure of only [3H]methanol that had been released during the demethylation incubation. Therefore, the fraction of peptide ester remaining was the total radioactivity minus the [3H]methanol released divided by the total radioactivity.

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**Peptide Isoaspartyl a-Methyl Esters**—The spontaneous rates of demethylation of the esters of many methylated erythrocyte proteins were comparable to that of the synthetic peptide ester studied here and were thus rapid on a physiological time scale (Table II, Refs. 18, 19, 22). The rapid spontaneous demethylation seems likely to result from demethylation mechanisms that involve the intermediate formation of intramolecular 5-membered rings. These derivatives include peptide succinimides (16-19, 22), anhydrides (22), or 5(4H)-oxazolones (23). In the present case, it is likely that facile formation of the succinimide product (Figs. 2 and 5) can account for the demethylation rate. A succinimide intermediate also appears to be the demethylation product of an a-methyl ester of deamidated adrenocorticotropic hormone (19). Although the formation of these intermediates has not been directly demonstrated from erythrocyte protein methyl esters, the rates of protein demethylation were consistent with intermediate formation (16-18, 22). It should be noted, however, that the rate of demethylation of at least one class of erythrocyte membrane protein methyl esters was about 375 times slower than the rate of the synthetic peptide methyl ester (Table II). Perhaps three-dimensional restrictions in protein conformation, or the presence of bulky residues nearby the aspartyl methyl ester, can slow the demethylation rate or restrict the utilization of different chemical demethylation pathways.

On the other hand, the spontaneous demethylation rate of
the bacterial chemoreceptor proteins methylated at several L-glutamyl residues was much less than even the slowest class of erythrocyte membrane protein methyl esters. For example, no demethylation of L-glutamyl residues in chemoreceptor proteins could be detected after 3 days at pH 7.2 at room temperature (24). For these proteins, the only mechanism for spontaneous hydrolysis may be direct attack of the ester by water or hydroxide ion. It has already been shown that the rate of formation of glutarimide is much less than the rate of succinimide formation for corresponding model peptides (16–18). Other factors such as sequence and three-dimensional structure could also play a role in the slower spontaneous demethylation seen for methylated bacterial chemoreceptor proteins.

Previous studies have measured the rate of hydrolysis of various methylated peptides (18). For some of these compounds such as aspartic acid β-methyl ester or N-benzyloaspartic acid β-methyl ester, the demethylation rate is similar to that of methyl propionate and is probably equal to the measured hydrolysis rate. However, for model compounds such as N-benzyloaspartic acid β-methyl ester glycinamide and the L-isohexapeptide methyl ester studied here, the net hydrolysis reaction appears to involve two steps, initial formation of succinimide and hydrolysis of the imide. The hydrolysis rate previously determined for N-benzyloaspartyl-β-methyl ester glycinamide by acid production in a pH-stat apparatus then measures the rate of imide opening and not that of the actual demethylation reaction (18, Table III). For the hexapeptide investigated here, the rate of succinimide opening was found to be 40 times slower than the rate of demethylation (Table I). For the various synthetic peptide esters studied by Bernhard et al. (16, 17), the rate of succinimide formation (demethylation).

What Role Does the Imide Play in the Metabolism of Damaged Proteins?—This work, along with that of Johnson and Aswad (19), suggests that enzymatically formed peptide and protein methyl esters in eucaryotic cells may be initially converted into succinimides by spontaneous demethylation.

**TABLE II**

| Methylated compound | t1/2 min |
|---------------------|---------|
| **In vitro methylated erythrocyte membranes** | |
| Fast 20% | 6 |
| Middle 30% | 35 |
| Slow 50% | 350 |
| **In vivo methylated erythrocyte membranes** | |
| Bands 2.1 and 3 | |
| Fast 50% | 90 |
| Slow 50% | 1500 |
| Band 4.1 | |
| Fast 60% | 60 |
| Slow 40% | 1500 |

*Methylated membranes are from trypsin-treated erythrocytes. Data are taken from Tewhiller and Clarke (18). *Data are taken from Barber and Clarke (10).
TABLE III

Comparison of demethylation and hydrolysis half-times of various methyl esters at pH 7.4 and 37 °C

|                  | Demethylation half-time* | Hydrolysis half-time* |
|------------------|--------------------------|-----------------------|
| l-Isohexapeptide methyl ester | 4                        | 180                   |
| ACTH*-isooaspartyl peptide methyl ester | 7.2                     | 252                   |
| N-Benzyl-aspartyl-β-methyl ester glycylamide | 100                     |                       |
| N-Benzyl-aspartic acid-β-methyl ester | 50,000                  |                       |
| Aspartic acid β-methyl ester | 1,400                   |                       |
| N-Benzyl-glutamyl-γ-methyl ester glycylamide | 1,000              |                       |
| N-Benzyl-glutamic acid-γ-methyl ester | 22,000                  |                       |
| Glutamic acid γ-methyl ester | 1,600                   |                       |
| Enzymatically methylated ovalbumin | 14                      |                       |

* Demethylation is the loss of the methyl group either by direct hydrolysis or formation of a succinimide or glutarimide ring.
* Hydrolysis refers to the step involving the uptake of water or hydroxide ion at the methyl ester itself or at the succinimide or glutarimide intermediate.
* Data for the l-isoheptapeptide methyl ester are taken from this work; other data (except as noted) are taken from Terwilliger and Clarke (18).
* ACTH, adrenocorticotropic hormone.
* Data of Johnson and Aswad (19).

reactions. Johnson and Aswad (19) detected no enhancement of the demethylation rate of the adrenocorticotropic hormone methyl ester derivative by purified protein carboxyl methyltransferase, and we have shown in this work that cytosolic extracts of erythrocytes, which might be expected to contain esterase activities, do not enhance the rate of demethylation of the synthetic hexapeptide ester. These results indicate, at least for the peptides under investigation, that the succinimide product may be relatively stable at physiological pH (Ref. 19, Table I). The most pressing questions now involve the fate of peptide and protein imides in eucaryotic cells. More specifically, we can ask whether subsequent steps are also nonenzymatic or if there is an enzyme that recognizes the imide and performs a reaction that perhaps restores it to its original configuration. In preliminary studies, we have incubated the l-succinimide hexapeptide in cytosolic extracts of erythrocytes. We do detect a rapid loss of the succinimide, but have not been successful in detecting either normal or isooxapetyl peptide products by HPLC. It appears that cytosolic peptidase activities may complicate the metabolism of this peptide. The addition of protease inhibitors (pepsatin, leupeptin, and phenylmethylsulfonyl fluoride) or ATP, ammonium ion, or glutamine does not appear to change the rate of loss of succinimide or the product distribution that we measure by HPLC (data not shown).

It is certainly possible that an esterase exists that does not recognize the hexapeptide ester which is not normally present in the red cell (although the l-isoheptapeptide was recognized by the cellular methyltransferase). Nevertheless, there was also no evidence for an esterase activity from studies of the demethylation of erythrocyte membrane proteins methylated in intact cells (23). If the product of demethylation of protein methyl esters in intact cells is, in fact, the imide, this intermediate may then appear twice along the damage-repair pathway, because the imide is a probable intermediate in the pathway of the deamination of asparaginyl residues to isoaspartyl residues (4, 25). If the imide formed in demethylation is an intermediate along a repair pathway (3–5), and is recognized by the putative repair enzyme, why is the first imide in the overall pathway not recognized by this repair enzyme? Although this question cannot be addressed experimentally at this time, it is possible that the two imides are not identical within the cell. For example, there could be differences in the conformation of the adjacent amino acids when the succinimide was formed by deamination of asparagine residues or when it was formed by the demethylation of α-methyl esters of l-isoaspartyl residues. It is also possible that a repair enzyme does not exist and that an efficient repair process occurs by completely chemical means. For various peptide succinimides, including the hexapeptide studied here, hydrolysis results in a product of about 75% isooxapite and 25% normal peptide (19, 26). Thus, in a population of cellular isoaspartyl residues that are methylated and then form imides, each cycle of demethylation, methylation, and ring opening would result in the conversion of about 25% of the isoaspartyl residues at the beginning of the cycle to normal aspartyl residues. In this way, repetitive methylation/demethylation cycles would eventually quantitatively restore all methylatable l-isoaspartyl residues to normal aspartyl residues.

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