Demethylation of Protein Carboxyl Methyl Esters: A Nonenzymatic Process in Human Erythrocytes?†

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ABSTRACT: We have compared the demethylation rate of protein carboxyl methyl esters from isolated human erythrocyte membranes with the corresponding rate of metabolic turnover of these same methyl groups in the intact erythrocyte. Surprisingly, the apparent spontaneous demethylation of these membrane protein methyl esters was significantly faster at physiological pH than the corresponding rate determined by pulse–chase analysis of intact cells incubated with L-[*methyl*-^{3}H]methionine. Readdition of erythrocyte lysate to purified membranes did not increase the rate of demethylation, as might be expected if there were cytosolic or membrane-bound protein methylesterase activity, but resulted instead in an apparent stabilization of these methyl esters. Thus, the metabolic lability of these protein methyl esters in intact cells may be quantitatively explained by spontaneous, rather than enzymatic, demethylation reactions. A model is presented in which a rapid but nonenzymatic intramolecular demethylation reaction results in the formation of a polypeptide imide or anhydride intermediate. The metabolic fate of these hypothetical intermediates is unknown but may lead to the repair or degradation of protein D-aspartyl and L-isoaspartyl residues, which appear to be the substrates for the initial transmethylation reaction.

One of the many ways by which proteins can be covalently modified is by the enzymatic transfer of a methyl group from the cellular methyl group donor AdoMet[1] onto acidic residues of proteins forming methyl esters. At least two general hypotheses have been proposed for the physiological function of protein carboxyl methylation in a eucaryotic cell such as the human erythrocyte. One hypothesis proposes that this post-translational covalent modification regulates the activity of the methyl-accepting substrate in analogy to the methylation reactions involved in bacterial chemotaxis (Springer et al., 1979). An alternative hypothesis is that the methylation reaction is not involved in the regulation of protein function but instead recognizes damaged proteins at D-aspartyl and L-isoaspartyl residues for further metabolism (Clarke & O'Connor, 1983; Clarke, 1985a,b).

If protein carboxyl methylation functions in a regulatory capacity in eucaryotic cells, then, even though esters of this kind hydrolyze rapidly at physiological pH (Terwilliger & Clarke, 1981), the degree of protein modification could be more finely controlled in response to physiological stimuli if the demethylation reaction was enzymatically catalyzed. On the other hand, if damaged proteins containing D-aspartyl residues or L-isoaspartyl linkages are carboxyl methylated as the first step in a "repair" process (McFadden & Clarke, 1982; Murray & Clarke, 1984), then the enzymatic coupling of demethylation with repair could serve to thermodynamically drive the repair reaction to completion. Thus, theoretical considerations stemming from both functional models suggest the possible existence of a protein demethylation activity in the human erythrocyte.

In chemotactic bacterial cells, a cytosolic protein methyl esterase has been purified and characterized that catalyzes the demethylation of L-glutamyl γ-methyl esters on membrane chemoreceptors (Stock & Koshland, 1978; Snyder et al., 1984). In mammalian cells, an activity that catalyzes the demethylation of gelatin, previously methylated in vitro with the protein carboxyl methyltransferase from erthrocytes, has been identified in kidney (Gagnon, 1979; Chene et al., 1982; Gagnon et al., 1984) and in neutrophils (Venkatasubramanian et al., 1980). However, the pH optimum of the kidney demethylase is about 4, and very little activity is detected at physiological pH values (Gagnon et al., 1984). Furthermore, the turnover number of the purified kidney enzyme for the ^{3}H-methylated gelatin substrate under optimal conditions is low [estimated at about 0.004 min^{-1} from purification data (Gagnon et al., 1984); the comparable value for the bacterial enzyme is about 60 times higher (Snyder et al., 1984)]. Considering these latter results, as well as the finding that this activity is present at very low to undetectable levels in many other mammalian tissues including brain (Gagnon, 1979; Chene et al., 1982; Aswad & Deight, 1983), the function of this enzyme in the demethylation of the physiologically carboxyl methylated proteins in the intact cell is unclear.

In this study we have attempted to identify a protein carboxyl methylesterase in human erythrocyte cytosol by using the endogenous, natural erythrocyte membrane protein methyl esters (Freitag & Clarke, 1981) as potential substrates. The results suggest that the metabolic lability of these methyl groups in intact erythrocytes is due to spontaneous, nonenzymatic hydrolysis.

Experimental Procedures

Cell Labeling with L-[*methyl*-^{3}*H*]*Methionine.* Human erythrocytes were obtained from healthy volunteers and

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ACTH, adrenocorticotropic hormone.

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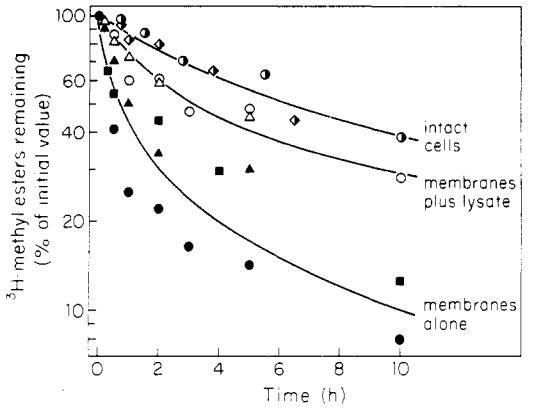

FIGURE 1: Demethylation of ^{3}H-labeled membrane protein methyl esters in intact cells, purified membranes in buffer, and purified membranes in erythrocyte lysate. After incubation of intact cells with L-[*methyl*-^{3}H]methionine as described under Experimental Procedures, membranes were purified by procedure A from individual 1 (triangles), by procedure B from individual 2 (circles), and by procedure C from individual 3 (squares). These purified membranes were incubated at 37 °C with 1 volume of either 50 mM Hepes, 115 mM NaCl, and 5 mM PMSF, pH 7.4 (closed symbols), or erythrocyte lysate (pH 7.4) as a source of cytosolic protein (open symbols). As a function of time, 30-μL aliquots were removed, membranes were washed in 2.7 mL of 5 mM buffer (using the same wash procedure used previously in their purification), and the [^{3}H]methyl esters remaining were determined by SDS gel electrophoresis as described under Experimental Procedures. Half-open symbols represent radioactive membrane protein methyl esters remaining after L-methionine chase of intact cells. After pulse-labeling as described under Experimental Procedures, these cells were washed 2 times with a 30-fold volume excess of 50 mM sodium phosphate–92 mM NaCl, pH 7.4, to remove residual L-[*methyl*-^{3}H]methionine. The label was then chased by incubation of cells at 37 °C in the same medium containing 0.3% D-glucose and 40 μM L-methionine at a 35% hematocrit for individual 2 (◑) or 100 μM L-methionine at a 0.7% hematocrit for individual 4 (right-solid diamond). As a function of time after the addition of L-methionine, aliquots were removed, cells pelleted by centrifugation, membranes isolated by procedure B (◑) or by procedure C (right-solid diamond), and the number of ^{3}H-methyl esters determined by SDS gel electrophoresis as described under Experimental Procedures. Summary: (●, ▲, ■) purified membranes in pH 7.4 buffer; (○, △) purified membranes in cytosol; (◑, right-solid diamond) pulse–chase analysis in intact cells.

washed 3 times in at least a 30-fold volume excess of 5 mM sodium phosphate–150 mM NaCl, pH 7.4. ^{3}H-Methylated membranes were prepared by incubating these cells at a 28% hematocrit with 6 μM L-[*methyl*-^{3}H]methionine (New England Nuclear, sp act. 80 Ci/mmol) for 3.5 h at 37 °C in 5 mM sodium phosphate, 150 mM NaCl, and 16 mM D-glucose, pH 7.4. The cells were then collected by centrifugation at 3000*g* for 5 min, and either the ^{3}H-labeled membranes were immediately purified by one of the procedures described below, or the cells were "chased" with excess L-methionine (see legend to Figure 1).

Preparation of Purified Erythrocyte Membranes. One of three procedures was followed. The level of protein methylation was essentially identical in all preparations, but there were differences in the amount of hemoglobin associated with the membrane.

Procedure A. Red cells were lyzed in at least a 30-fold volume excess of 5 mM sodium phosphate, pH 6.8, at 0 °C and washed 3 more times with at least a 30-fold volume excess of the same buffer. Because buffer at this pH is not efficient at extracting tightly bound hemoglobin (Dodge et al., 1963), the final membrane pellet was still pink.

Procedure B. This method was similar to procedure A but lysis was performed in 5 mM sodium phosphate, pH 8.0. After 1 min at 0 °C to allow complete lysis, the lysate was acidified to pH 6.8 with 46 mM sodium citrate buffer (pH 5.4) as described (Terwilliger & Clarke, 1981). Membranes were then pelleted by centrifugation and washed 2 more times by the same procedure. The final membrane pellet was slightly cream colored.

Procedure C. This method was similar to procedure A except that cells were lyzed in 5 mM sodium phosphate, pH 8.0, at 0 °C and pelleted without acidification of the lysate. Membranes were then washed 2 more times in the lysis buffer. The final membrane pellet was white.

Analysis of ^{3}H-Labeled Protein Methyl Esters. Except where stated otherwise, the radioactivity in protein methyl esters was determined by SDS electrophoresis in 7.5% polyacrylamide gels at pH 2.4 followed by autofluorography as described (Barber & Clarke, 1983). In this way, significant proteolysis would have been readily detected on the Coomassie-stained gel. It has been demonstrated that the total radioactivity in membrane proteins separated by SDS gel electrophoresis in this manner is at least 90% in the form of methyl esters (Freitag & Clarke, 1981).

Preparation of Erythrocyte Lysate. Erythrocytes were obtained and washed with isotonic buffer at either pH 6.0 (for the experiment in Figure 2) or pH 7.4 (for all other experiments). Cell lysis was induced by one cycle of freeze-thawing of packed erythrocytes. Lysis was essentially complete as confirmed by dilution with isotonic buffer and centrifugation. The pH was measured after dilution of the lysate with 10–100 volumes of deionized water.

RESULTS

The rate of demethylation of erythrocyte membrane protein carboxyl methyl esters was determined in intact cells and in preparations of purified membrane fractions (Figure 1). The rate of spontaneous demethylation of a purified, cytosol-free, membrane fraction in pH 7.4 buffer was rapid ($t_{1/2}$ = 45 min). Because of this apparent chemical lability at physiological pH, it was possible that the metabolic demethylation of membrane protein methyl esters observed for intact erythrocytes during pulse–chase analysis [cf. Figure 1 and Freitag & Clarke (1981)] could be a nonenzymatic reaction. In fact, comparison of the relevant data in Figure 1 (spontaneous demethylation in pH 7.4 buffer vs. the metabolic turnover in intact cells) indicates that spontaneous hydrolysis can more than account for the metabolic turnover of the methyl groups observed in intact cells. The chase of radioactivity from cellular [^{3}H]-AdoMet has been shown to be rapid and complete under these conditions (Barber & Clarke, 1984).

To determine if a protein carboxyl methylesterase activity exists in erythrocyte cytosol, undiluted cell lysate was added to these ^{3}H-labeled, purified membranes. An increase in the rate of protein demethylation in the presence of this extract would be positive evidence for the existence of an erythrocyte protein methylesterase activity. However, the opposite result was obtained (Figure 1). The addition of erythrocyte lysate at pH 7.4 to purified membranes tended to stabilize the spontaneous demethylation observed for the purified membranes alone in pH 7.4 buffer. The rate of demethylation in the presence of lysate more closely resembled the corresponding metabolic rate observed for intact cells.

To verify that the loss of radioactivity from protein [^{3}H]-methyl esters observed in Figure 1 is due to spontaneous, base-catalyzed demethylation, the loss of [^{3}H]methyl esters of purified membrane proteins was measured as a function of pH.[2] The demethylation rates for erythrocyte membrane

Table I: Temperature and pH Dependence of Demethylation of Purified Membrane Protein [^{3}H]Methyl Esters Obtained from Intact Cells[a]

conditions	approximate time for 50% demethylation (min) 37 °C	0 °C
pH 10.5	3	120
pH 9.0	15	900
pH 7.4	45[b]	6000[c]

[a] After incubation of intact erythrocytes with L-[*methyl*-^{3}H]-methionine as described under Experimental Procedures, membranes were purified by procedure A. These membranes were incubated at either 0 or 37 °C in one of the following buffers: 50 mM Hepes, 115 mM NaCl, 5 mM PMSF, pH 7.4; 50 mM borate, 115 mM NaCl, 5 mM PMSF, pH 9.0; 50 mM glycine, 115 mM NaCl, 5 mM PMSF, pH 10.5. As a function of time, aliquots were removed, and the radioactive methyl esters remaining were determined by gel electrophoresis as described under Experimental Procedures. [b] Taken from data for purified membranes in Figure 1. [c] Taken from data for purified membranes in Figure 2.

Table II: Comparison of the Rate of Demethylation of Protein Methyl Esters of Erythrocyte Membranes with Soluble Protein and Small Molecule Methyl Esters[a]

methyl ester	$t_{1/2}$ of demethylation at 37 °C (min) pH 7.4	pH 9.0	pH 10.5
(A) protein and peptide esters			
red blood cell membrane proteins[b]	45	15	3
deamidated ACTH[c]	7.2		
ovalbumin[d]	14		
glucagon[e]	55	5	
calmodulin[f]	120	20	2
L-Val-L-Tyr-L-Pro-L-isoAsp(α-methyl ester)-Gly-L-Ala[g]	4		
N-benzoyl-L-Asp(β-methyl ester)-Gly-NH_2[h]	95[i]	3.1[i]	
N-benzoyl-L-Glu(γ-methyl ester)-Gly-NH_2[h]		50[i]	2[i]
(B) *N*-benzoyl amino acid esters			
N-benzoyl-L-Asp(β-methyl ester)[h]	50 000[j]	1550	65
N-benzoyl-L-Glu(γ-methyl ester)[h]	22 000	1300	61

[a] Rates are measured for the loss of [^{3}H]methyl groups from the esters in buffer at the indicated pH in all cases except for the benzoyl esters where the rate of hydrolysis in water is measured in a pH-stat apparatus. Methylated derivatives of ovalbumin, glucagon, and calmodulin were prepared with the human erythrocyte carboxyl methyltransferase: the ACTH derivative was prepared with the bovine brain enzyme. For the benzoylglycylamide derivatives, where intermediate succinimide formation is a likely reaction, the rate of the initial demethylation reaction is probably about 10-fold greater, and the corresponding $t_{1/2}$ values should be lower by this approximate factor [cf. Bernhard et al. (1962), Bernhard (1983), and Johnson & Aswad (1985)]. [b] Data from Table I. [c] Johnson & Aswad (1985). [d] S. Clarke (unpublished data). [e] K. L. Oden and S. Clarke (unpublished data). [f] L. S. Brunauer and S. Clarke (unpublished data). [g] E. D. Murray, Jr., and S. Clarke (unpublished data). [h] Terwilliger & Clarke (1981). [i] These data reflect the hydrolysis reaction, probably of a succinimide intermediate (see above). [j] Extrapolated value.

proteins in Table I are similar to those previously measured for the spontaneous demethylation of polypeptides enzymatically methylated by the erythrocyte or brain carboxyl methyltransferase as well as for two synthetic methyl ester peptides that contain amide linkages on the α-carboxyl group of the carboxylic acid residue (Table II).

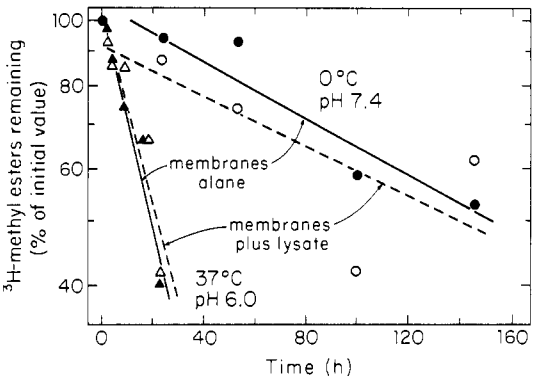

FIGURE 2: Effect of erythrocyte lysate on demethylation of membrane proteins at pH 6.0 or at 0 °C. Intact erythrocytes were incubated with L-[*methyl*-^{3}H]methionine, and membranes were isolated either by procedure B (circles) or by procedure C (triangles) as described under Experimental Procedures. Membranes were then incubated either at 37 °C (triangles) or at 0 °C (circles) in 1 volume of either 218 mM MES, pH 6.0 (▲), 50 mM Hepes, 115 mM NaCl, and 5 mM PMSF, pH 7.4 (●), or packed erythrocytes that had been previously washed in either of these buffers and lysed by freeze-thawing as a source of lysate at pH 6.0 (△) or pH 7.4 (○). Remaining [^{3}H]methyl esters were measured as a function of time by either the SDS gel assay described under Experimental Procedures (pH 7.4, 0 °C) or a volatilization assay (pH 6.0, 37 °C) involving the transfer of the [^{3}H]methanol released by base hydrolysis of trichloracetic acid precipitated protein into scintillation counting fluid as described (MacFarlane, 1984). Lines were drawn from a least-squares fit of the data.

It was possible that a methylesterase activity existed but was not detected because its velocity was similar to the rate of spontaneous demethylation. In an attempt to detect such an activity, it was necessary to seek conditions under which the nonenzymatic reaction could be minimized while leaving the hypothetical enzymatic activity unaffected. Since the data in Table I confirm the expected lability for protein methyl esters at higher pHs, a pH below physiological pH was chosen to limit spontaneous demethylation. The data in Figure 2 indicate that although the nonenzymatic rate of protein demethylation of purified membranes is much lower under these conditions (compare to the data in Figure 1), the presence of lysate proteins at pH 6.0 is ineffective at accelerating the demethylation rate in pH 6.0 buffer. Since it was possible that a protein methylesterase activity might not be active at nonphysiological pHs and since the data in Table I also reveal a marked temperature dependence in the rate of spontaneous demethylation, the effect of lysate on demethylation at pH 7.4 was determined at 0 °C (Figure 2). These conditions also fail to indicate a significant acceleration of demethylation in the presence of lysate at 0 °C compared to the spontaneous demethylation in buffer at 0 °C (Figure 2).

Discussion

Previous studies of mammalian protein methylesterase activity utilized nonphysiological methyl ester substrates (Gagnon, 1979; Chene et al., 1982; Gagnon et al., 1984). In our search for an erythrocyte protein methylesterase, we took advantage of the fact that the physiological rate of turnover of membrane methyl esters formed in intact cells could be measured in pulse–chase experiments [cf. Figure 1; Barber & Clarke (1983)] and that the demethylation rate of these same esters could be quantitated with the isolated membrane fraction. Thus, the spontaneous rate of demethylation of these physiologically methylated proteins could be measured and

[2] It should be noted that the rates of demethylation of red cell membrane proteins (and to a lesser extent the other enzymatically methylated polypeptides such as glucagon or calmodulin) are not directly proportional to the hydroxide ion concentration, as would be expected for a strictly base-catalyzed reaction. This result may reflect the participation of a pH-independent demethylation mechanism and/or changes in the demethylation rate that relate to pH-dependent changes in protein structure.

compared to their actual metabolic lability in intact cells.

The apparent lack of protein methylesterase activity reported here suggests that protein demethylation, the corresponding demodification reaction of protein carboxyl methyl esterification, is a nonenzymatic process for human erythrocyte membrane proteins. However, there are several possible reasons why a protein methyl esterase activity might be difficult to detect. First, the major sites of carboxyl methylation in intact cells, used here as potential methylesterase substrates, might be resistant to the action of a cellular methylesterase. The degree of modification of these major carboxyl methylated proteins may, in fact, be high because these protein methyl esters are not rapidly demethylated by the protein methylesterase. Second, the membrane fraction itself may contain a tightly bound protein methylesterase activity. We feel this latter possibility is unlikely because the lysate fraction, containing membranes, does not increase the rate of demethylation. Additionally, if the membrane fraction contained an esterase, one would expect that the intrinsic rate of demethylation of purified membrane protein esters would be more rapid than those rates measured for purified membrane protein and methyl esters. Examination of the data in Table II (part A) indicates that this does not appear to be the case. Furthermore, one might expect that a membrane-bound methylesterase would have difficulty encountering its many cytoskeletal and cytosolic substrates because its three-dimensional diffusion would be restricted. Finally, we cannot rule out the presence of an endogenous inhibitor that effectively masks the esterase activity.

Possible mechanisms of protein demethylation are summarized in Figure 3. Reaction 1, the straightforward nonenzymatic hydrolysis reaction, does not appear to contribute significantly to the demethylation rate because reactions of this type are much slower than the rate of spontaneous demethylation observed in this study. For example, $t_{1/2}$ of hydrolysis of methyl propionate at pH 7.4 and 37 °C is 5600 min; the extrapolated comparable value for *N*-benzyl-L-aspartic acid β-methyl ester is 50 000 min (Terwilliger & Clarke, 1981). These half-times are thus about 100–1000 times longer than the measured values for red cell membrane esters (Table I).

To explain the rapid spontaneous demethylation of erythrocyte protein methyl esters, intramolecular mechanisms are proposed (Figure 3, reactions 2 and 3) in which the juxtaposition of protein functional groups results in the formation of succinimide (Battersby & Robinson, 1955; Bernhard et al., 1962; Bernhard, 1983) and/or anhydride intermediates in the hydrolysis reaction. Here, the nucleophile that attacks the carbonyl carbon of the ester is not water or hydroxide ion but the nitrogen and/or carbonyl oxygen atom of the carboxyl peptide bond (Figure 3). These latter reactions result in the five-membered succinimide and anhydride rings, respectively. Thus, demethylation (the loss of methanol) and hydrolysis (the covalent addition of water to the structure) may be chemically and temporally distinct events (Figure 3). The imide mechanism of demethylation is not possible for compounds such as *N*-benzoylaspartyl or *N*-benzoylglutamyl β- or γ-methyl ester where the α-carboxyl group is not involved in an amide bond; this fact may explain the large differences in the rates of demethylation observed between the peptide and protein methyl esters and these compounds in Table II.

The relative rate of each of the intramolecular demethylation mechanisms (reaction 2 or 3) for a particular methylated residue in a protein will depend on the nucleophilicity of the peptide oxygen or nitrogen atom and on the relative proximity of these nucleophiles. Thus, the three-dimensional structure

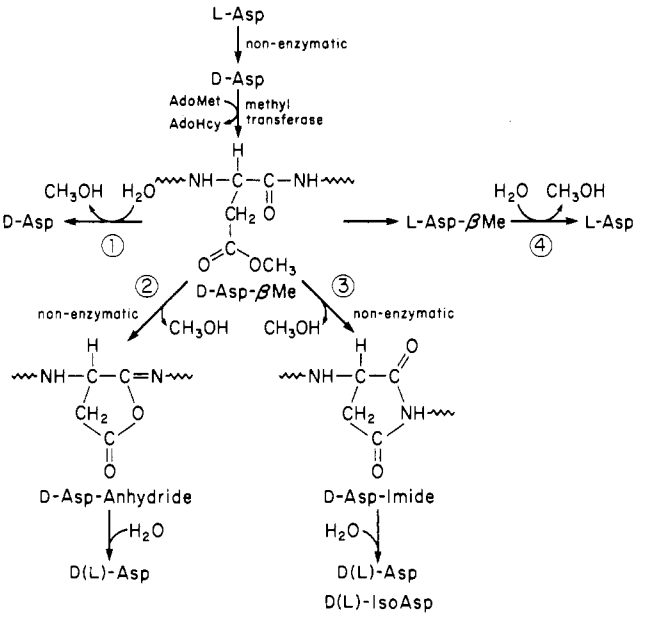

FIGURE 3: Hypothetical mechanisms of nonenzymatic protein demethylation. Although the scheme drawn above is for D-aspartyl residue methylation, a similar set of reactions could be drawn for L-isoaspartyl methylation. In the latter case, the intermediate of reaction 2 would be the L-isoAsp anhydride while that of reaction 3 would be the L-succinimide. The relative rates of reaction 2 and 3 have not been established for erythrocyte membrane proteins. One factor that would be expected to be important in determining the fate of the aspartyl β-methyl ester is the dihedral angle ψ between the α-carbon and the carbonyl carbon of the peptide bond. For D-aspartyl β-methyl ester residues, maximal anhydride formation would be expected at a value of about −60°, where the peptide carbonyl oxygen is adjacent to the carbonyl carbon of the ester. This conformation would be found in an α helix. On the other hand, the peptide nitrogen on the carboxyl side is adjacent to the ester carbonyl when the angle ψ is about 120°. This latter conformation would be found in a β structure and would appear to favor succinimide formation from the D-aspartyl α-methyl ester. The position of the carboxyl peptide nitrogen and carbonyl oxygen relative to the ester carbonyl would also be important in determining whether an anhydride or imide intermediate would be favored as demethylation products for L-isoaspartyl α-methyl esters. For isoaspartyl α-methyl esters, an additional intermediate, a 5(4*H*)-oxazolone, may also be formed (Benoiton, 1983).

of the protein in the region of the methylated residue may be an important factor in the demethylation reaction. For example, a D-aspartic acid β-methyl ester residue in an α helix would be more likely to form an anhydride intermediate while such a residue in a β structure might be expected to form a succinimide intermediate (see Figure 3 legend). The rapid nonenzymatic formation of succinimide from a methylated isoaspartyl derivative of adrenocorticotropic hormone has been recently described by Johnson & Aswad (1985), and similar results have been obtained for the synthetic peptide L-Val-L-Tyr-L-Pro-L-isoAsp(α-methyl ester)-Gly-L-Ala (E. D. Murray and S. Clarke, unpublished data). For peptides like these, where little secondary or tertiary structure exists in solution, the geometrical considerations may be less important than for methylated residues in relatively less flexible protein molecules.

The fate of the succinimide and/or anhydride intermediates proposed to occur as a result of reactions 2 and 3 in Figure 3 is unknown. If the only substrates for the protein carboxyl methyltransferase are L-isoaspartyl and D-aspartyl residues (Aswad, 1984; Murray & Clarke, 1984, McFadden & Clarke, 1982), a mixture of D- and L-succinimide, D-anhydride, and L-isoanhydride residues would be formed. These residues could undergo a variety of enzymatic (or possibly nonenzymatic) reactions that include racemization or epimerization at the α-carbon, hydrolysis to the aspartyl and isoaspartyl residues,

amidation, cross-linking, or peptide bond cleavage (McFadden & Clarke, 1982; Murray & Clarke, 1984; Aswad, 1984; Johnson & Aswad, 1985; Clarke, 1985ab). For example, a D-succinimide residue might be converted to a L-aspartyl residue in a repair reaction or may be a substrate for a protease. The identification of specific succinimide or anhydride residues will allow the pathway of the cellular metabolism of these protein derivatives to be elucidated.

The fact that purified [^{3}H]carboxyl-methylated membrane proteins apparently lose their methyl groups more slowly in the presence of erythrocyte lysate at pH 7.4 than in pH 7.4 buffer (see Figure 1) suggests that these major methylated proteins are somehow protected from demethylation in the intact cell, perhaps by binding to a cytosolic protein. Such a protein might be involved in the subsequent metabolism of these methylated proteins by performing some slow, rate-limiting reaction (perhaps a protein repair reaction such as the one described by reaction 4 in Figure 3) before allowing the methylated protein to lose its methyl ester. Similarly, the protein might direct the hydrolysis reaction to avoid an imide intermediate mechanism and the subsequent polypeptide chain isomerization that results from spontaneous hydrolysis of the type observed for reaction 3 in Figure 3.

Registry No. L-Val-L-Tyr-L-Pro-L-isoAsp(α-methyl ester)-Gly-L-Ala, 97170-87-1; glucagon, 9007-92-5.

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