Enzymatic Protein Carboxyl Methylation at Physiological pH: Cyclic Imide Formation Explains Rapid Methyl Turnover†

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ABSTRACT: At pH 7.4, 37 °C, bovine brain protein carboxyl methyltransferase transiently methylates deamidated adrenocorticotropin. The methylation occurs at the α-carboxyl group of an atypical β-carboxyl-linked isoaspartyl residue (position 25). Several lines of evidence indicate that the immediate product of demethylation is an aspartyl cyclic imide involving positions 25 and 26. The evidence includes (1) the rapid rate of methyl ester hydrolysis, which is consistent with intramolecular catalysis, (2) the inability of the demethylated product to be remethylated, (3) the charge of this product, and (4) its rate of breakdown. The eventual hydrolysis of the cyclic imide produces a 30/70 mixture of peptides containing either α- or β-carboxyl-linked aspartyl residues, respectively. Cyclic imide formation is nonenzymatic and can explain the unusual lability of mammalian protein methyl esters in general. These findings suggest that protein carboxyl methylation in mammalian tissues is not a simple on/off reversible modification as it apparently is in chemotactic bacteria. Carboxyl methylation may serve to activate selected protein carboxyl groups for subsequent longer lasting modifications, possibly subserving a role in protein repair, degradation, cross-linking, or some other as yet undiscovered alteration of protein structure.

Covalent protein modification plays an important role in a wide range of cellular processes including reversible regulation of enzyme/protein function, long-lasting modification of amino acid side chains (e.g., N-methylation of lysine or γ-carboxylation of glutamate), and protein degradation (Wold, 1981; Hershko & Ciechanover, 1982). Of the many protein modification systems described to date, the eucaryotic protein carboxyl methyltransferases (PCMTs)[1] are among the least well understood in terms of their biological function. This is in spite of the fact that this enzyme activity was first discovered 20 years ago (Axelrod & Daly, 1965) and is apparently ubiquitous in its tissue distribution (Diliberto & Axelrod, 1976; Paik & Kim, 1980; Trivedi et al., 1982). Lack of progress toward understanding the function of this enzyme can be attributed, at least in part, to a poor understanding of its natural substrates. Most proteins, especially denatured ones, can serve as methyl acceptors to some degree (Paik & Kim, 1980; Kim & Paik, 1971; Diliberto & Axelrod, 1974; Edgar & Hope, 1976; Kloog et al., 1980; Aswad & Deight, 1983; Clarke & O'Connor, 1983). Protein methylation both in vitro and in vivo generally occurs with a low stoichiometry (Kloog et al., 1980; Aswad & Deight, 1983; Clarke & O'Connor, 1983; Kim & Li, 1979), and the protein methyl esters formed are considerably more labile than free aspartate or glutamate methyl esters (Paik & Kim, 1980; Kim & Paik, 1976; Terwilliger & Clarke, 1981).

Recent studies on the methyltransferases from bovine brain (Aswad, 1984) and human erythrocytes (Murray & Clarke, 1984) have suggested an explanation for these unusual features of the mammalian methyltransferase reaction. These enzymes have been shown to selectively and stoichiometrically methylate the α-carboxyl group of a L-isoaspartyl residue, which results from deamidation of asparagine-25 in adrenocorticotropin (ACTH). Atypical isopeptide-linked aspartyl residues can occur in a variety of proteins as a result of spontaneous deamidation of certain aspargine residues, particularly those linked to the α-amino group of glycine (Gräf et al., 1970, 1971; Bornstein & Balian, 1977). A two-step mechanism (Figure 1) involving anchimeric assistance by the sterically unhindered glycine nitrogen to promote formation of a cyclic imide intermediate has been proposed (Bornstein & Balian, 1977) and is well supported by model studies (Sondheimer & Holley, 1954; Battersby & Robinson, 1955; Bodanszky & Kwei, 1978). The selective methylation of deamidation-dependent, atypical isoaspartyl residues can readily explain the low stoichiometry of methyl incorporation into protein acceptors (Aswad, 1984).

Selective methylation of glycine-linked isoaspartyl residues could also explain the unusual lability of the enzymatically formed protein methyl esters at physiological pH. Demethylation of these sites would be expected to proceed by the same mechanism that occurs during deamidation (Battersby & Robinson, 1955; Bodanszky & Kwei, 1978). Thus, the immediate product of demethylation (the cyclic imide) should be resistant to remethylation, a prediction that runs counter to the commonly held concept that carboxyl methylation is a simple reversible protein modification (Paik & Kim, 1980; Gagnon & Heisler, 1979; O'Dea et al., 1981). The formation of a stable cyclic imide has important implications for possible functions of the methyltransferase reaction. In this paper, we have investigated the methylation and demethylation of deamidated ACTH under conditions of physiological pH and temperature. The results indicate that carboxyl methylation does indeed lead to formation of a stable cycle imide.

Experimental Procedures

Purification of PCMT. The type I isozyme of PCMT was purified from bovine cerebral cortex as previously described (Aswad & Deight, 1983a). The enzyme had a specific activity of 12 000–16 000 pmol min^{-1} mg^{-1} at pH 6, 30 °C, with 5.0

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[1] Abbreviations: ACTH, adrenocorticotropin; AdoMet, *S*-adenosyl-L-methionine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; isoACTH, the form of deamidated ACTH that contains a β-carboxyl-linked isoaspartyl residue in position 25; PCMT, protein carboxyl methyltransferase.

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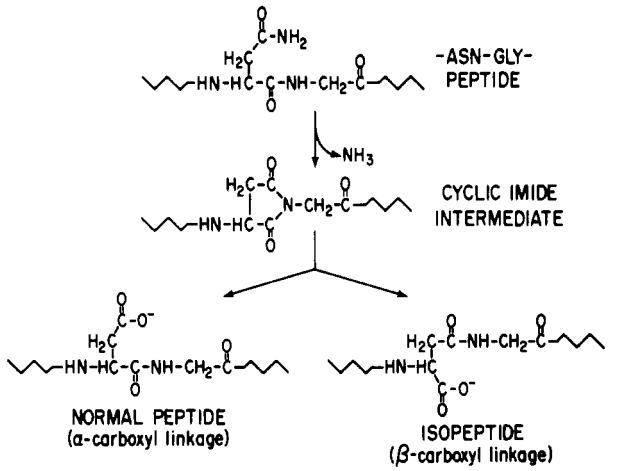

FIGURE 1: Mechanism of deamidation of ACTH. The deamidation of Asn-25 proceeds through a cyclic imide intermediate involving the α-amino group of Gly-26. Hydrolysis of the cyclic imide produces two products, one containing an α-carboxyl-linked Asp–Gly peptide bond and the other a β-carboxyl-linked isoAsp–Gly bond. The isopeptide and normal forms are generated in the ratio of ca. 70/30 (Murray & Clarke, 1984). The evidence for this mechanism has been reviewed by Bornstein & Balian (1977).

mg/mL γ-globulin (Sigma, G3500) as the methyl acceptor. Purity was estimated to be greater than 90% by gel electrophoresis in sodium dodecyl sulfate. The preparation was devoid of detectable endogenous substrate.

Purification of Native and Deamidated ACTH. Deamidated ACTH was generated by incubating highly purified porcine ACTH (Sigma, grade II, lot 102F-0781) for 3 h at 37 °C in 0.1 M NH_4OH. The deamidated and native components were isolated by chromatography on CM-cellulose as described elsewhere (Aswad, 1984).

Purification of S-Adenosyl-L-methionine. Unlabeled *S*-adenosyl-L-methionine (AdoMet; Sigma A7007) was purified by CM-cellulose chromatography by the method of Chirpich (1968) and was used to adjust the specific activity of the [*methyl*-^{3}H]AdoMet solutions. [*methyl*-^{3}H]AdoMet (5–15 Ci/mmol) was obtained from Amersham.

Methylation Reactions. Except where noted otherwise, reactions were carried out at pH 7.4, 37 °C, in a final volume of 50 μL containing 50 mM K-HEPES, 1 mM EGTA, 10 μM ACTH, 2.5 μM PCMT, and 200 μM [*methyl*-^{3}H]AdoMet (300–400 dpm/pmol). The latter component was added in 10 μL after all other components had been preincubated at 37 °C for 2 min. Blanks lacked ACTH and did not vary with the duration of incubation.

Reactions were stopped in one of two ways. To determine methyl groups incorporated into protein, reactions were terminated by the addition of 0.8 mL of ice-cold 10% trichloroacetic acid. A total of 20 μL of 25 mg/mL γ-globulin was added as a carrier. Precipitates were washed by repeated centrifugation and resuspension as previously described (Aswad & Deight, 1983a). Control experiments indicated that the overall recovery of ACTH was greater than 95%. Total carboxyl methylation was estimated by a method similar to that of MacFarlane (1984). Reactions were terminated by the addition of 50 μL of 0.22 M sodium borate, pH 10.2, 2% (w/v) sodium dodecyl sulfate, and 50% (v/v) methanol. This was followed by a 30-min incubation at 37 °C to facilitate the hydrolysis of the methyl esters. Tubes then sat at 4 °C for 1 h. They were centrifuged to remove condensation from the walls of the tubes, and the entire contents were pipetted onto filter paper (Whatman 3MM) lodged in the necks of 20-mL glass scintillation vials containing 8 mL of ACS scintillation fluid (Amersham). Vials sat for 3 h at room temperature, during which time diffusion of the labeled methanol reached completion. Filter papers were removed, and radioactivity was measured by scintillation counting. Recovery of methanol was determined in a series of control tubes by substituting [^{14}C]methanol (~20000 dpm) for [*methyl*-^{3}H]AdoMet. Recovery was 80%–85% and did not vary with duration of incubation at 37 °C, pH 7.4. Methylation reactions performed at varying pH used the following buffers: pH 7 and 8, 50 mM K-HEPES; pH 9, 50 mM potassium borate.

Analysis of Transiently Methylated IsoACTH: Isolation of the Cyclic Imide. Deamidated ACTH, consisting of ca. 70% isopeptide (isoACTH) and 30% normal peptide (see Figure 1), was incubated for 90 min at 37 °C in an 890-μL reaction mixture containing 50 μM peptide, 5 μM PCMT, 50 mM K-HEPES (pH 7.4), 1 mM EGTA, and 500 μM [*methyl*-^{3}H]AdoMet (60 dpm/pmol). The reaction was stopped by addition of 90 μL of 1.0 M acetic acid and then lyophilized. Gel filtration was carried out at 24 °C on a 1 × 30 cm column of Sephadex G-25 equilibrated with 50 mM ammonium acetate (pH 4.8), using a flow rate of 6 mL/h. Fractions containing the peptide were pooled, and half of this pool (~100 μg) was taken for cation-exchange HPLC as described below.

Cation-Exchange HPLC. Cation-exchange HPLC was performed on a 4.6 × 10 cm column of Aquapore CX-300 (Brownlee Labs, Santa Clara, CA). The column was equilibrated with 10 mM ammonium acetate, pH 6.7. Samples (10–200 μg of protein) were injected in a volume of 0.5–1.0 mL at a flow rate of 1.5 mL/min. After 5 min, a linear gradient to 500 mM ammonium acetate, pH 6.7, over 30 min was initiated. Elution continued at a flow rate of 1.5 mL/min. Absorbance of the effluent was monitored at 280 nm. Retention times refer to the time elapsed after gradient initiation.

Reversed-Phase HPLC. Reversed-phase HPLC was performed on a 4.6 × 10 cm column of Aquapore RP-300 (Brownlee Labs) fitted with a 3-cm guard column of the same material. The column was equilibrated with 0.1 M acetic acid. Methylated ACTH (200 μg) was injected in 2 mL at a flow rate of 1.5 mL/min. After 5 min, a linear gradient to 80% acetonitrile in 0.1 M acetic acid over 15 min was initiated. Absorbance was monitored at 280 nm. The methylated ACTH eluted with a retention time of 8.3 min and was identified by scintillation counting.

Protein Determination. ACTH was determined by the method of Bradford (1976) using dye reagent from Bio-Rad (Richmond, CA). Deamidated ACTH was used as a standard. PCMT was determined by the method of Lowry et al. (1957) using bovine serum albumin as a standard.

Reaction Modeling. The predicted time courses of ^{3}H-labeled peptide and [^{3}H]methanol levels shown in Figure 2 were calculated from the kinetic constants given in Table I and the set of differential equations defined by the reaction scheme shown in Figure 3. In the following equations, A = [isopeptide], B = [methyl isopeptide], C = [imide], D = [methanol], E = [normal peptide], and A_0 = [isopeptide] at time zero:

$$A_0 = A + B + C + E \quad (1)$$

$$dA/dt = k_3C - V_mA/(K_m + A) \quad (2)$$

$$dB/dt = V_mA/(K_m + A) - k_2B \quad (3)$$

$$dC/dt = k_2B - (k_3 + k_4)C \quad (4)$$

$$dD/dt = k_2B \quad (5)$$

$$dE/dt = k_4C \quad (6)$$

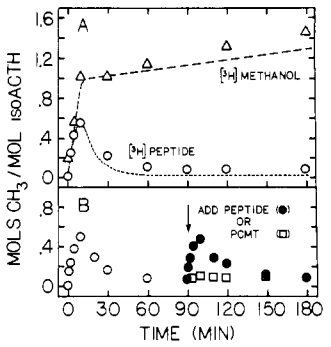

FIGURE 2: Kinetics of isoACTH methylation and methanol production. The methylation reaction was carried out at pH 7.4, 37 °C, as described under Experimental Procedures. Panel A shows the time courses of methyl incorporation into isoACTH measured by trichloroacetic acid preciptation (○) or total methanol recovered after stopping the reaction with pH 10 borate buffer (△). The broken curves indicate the predicted time courses for isoACTH methylation (- - -) and methanol recovery (— — —) on the basis of the scheme proposed in Figure 3 and the kinetic constants in Table I (see Experimental Procedures). Panel B shows a control experiment to verify that the methyltransferase activity is nearly constant throughout the incubation period. Methylation of isoACTH was determined as in (A) above. At 90 min, the amount of isoACTH or PCMT was doubled in parallel reaction tubes. These additions were made in volumes that increased the reaction volume by 10%. Addition of new peptide elicited a new burst of transient methylation that was nearly identical with the original burst, indicating that significant inactivation of PCMT or depletion of [^{3}H]AdoMet had not occurred.

Table I: Kinetic Constants for the Reaction Scheme Presented in Figure 3[a]

reaction step	constant	value
1	V_{max}	15 nmol min^{-1} mg^{-1}
	K_m	1.3 μM
2	k_2	96.3×10^{-3} min^{-1}
3	k_3	1.93×10^{-3} min^{-1}
4	k_4	0.825×10^{-3} min^{-1}

[a] V_{max} was estimated from the initial rate of methylation observed in Figure 2 with a correction for subsaturation of the enzyme. The K_m was taken from our previous study (Aswad, 1984) with a correction for the fact that deamidated ACTH consists of 70% isopeptide. The value for k_2 was calculated from the half-life of the methyl ester as described in the text ($t_{1/2}$ = 7.2 min). Values for k_3 and k_4 were derived from the linear portion of the peak II data in Figure 5 ($t_{1/2}$ = 4.2 h, k_{hydr} = 2.74×10^{-3} min^{-1}), with the additional knowledge that $k_3 + k_4 = k_{hydr}$ and that $k_3 = k_4 \times 7/3$.

The initial value problem was solved by numerical integration of the above equations with a fourth-order Runge–Kutta algorithm in 0.1-min time steps (Gear, 1971). The calculations were carried out by Kim Uyehara and Dr. Donald H. Perkel of the University of California, Irvine.

Results

Kinetics of IsoACTH Methylation and Methanol Production at Physiological pH and Temperature. To test the prediction that the immediate product of isoACTH demethylation is a cyclic imide, we studied the kinetics of methyl incorporation and methanol production at pH 7.4 and 37 °C. Imides of this type are relatively stable at neutral pH (Sondheimer & Holley, 1954; Battersby & Robinson, 1955; Bernhard et al., 1962). Consequently, the methylation of isoACTH under these conditions should appear largely irreversible. The level of methyl incorporation into isoACTH should initially increase and then fall to near zero as the original substrate is converted to the nonmethylatable cyclic imide. If, on the other hand, the methylation reaction were reversible, i.e., the demethylation product was the original substrate, then the methylation level of isoACTH should

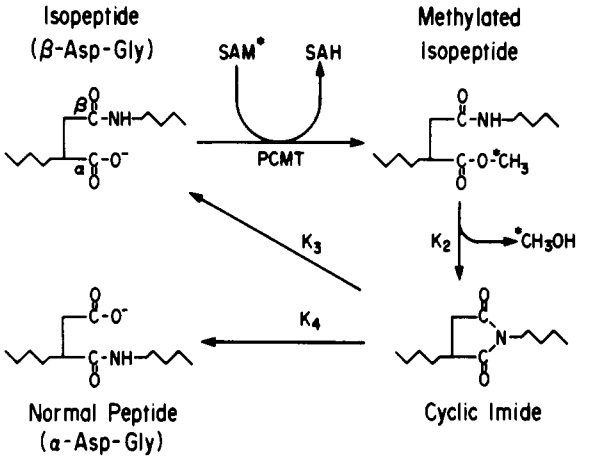

FIGURE 3: Proposed reaction scheme for the methylation and demethylation of isoACTH at pH 7.4. The radiolabel is identified by an asterisk (*). SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine. For details, see text.

gradually increase to a constant value representing an equilibrium between the rates of methylation and demethylation. Figure 2A shows the methylation kinetics measured in two ways. The lower curve shows [^{3}H]methyl incorporation into isoACTH and exhibits the biphasic shape expected for an irreversible cycle of methylation/demethylation. This interpretation assumes that the methyltransferase activity and supply of [*methyl*-^{3}H]AdoMet remained relatively constant during the reaction. As shown in Figure 2B, this was indeed the case.

The upper curve shows the total [^{3}H]methanol present in the reaction tube after stopping the reaction at the indicated times with a strongly alkaline solution. This curve represents the sum of any [^{3}H]methyl groups incorporated into isoACTH plus any previously incorporated methyl groups that had spontaneous hydrolyzed during the reaction. This curve is also biphasic, exhibiting a marked transition from rapid methanol production to slow methanol production coinciding with the peak of maximal methyl incorporation into isoACTH. The slow rate of methanol production after 10 min indicates that only a small amount of methylation is occurring during this period, even though the ACTH is largely unmethylated. This further supports the conclusion that the demethylation of the ACTH yields a product that is resistant to methylation.

Extrapolation of the slow phase of methanol production to zero time indicates that the isoACTH is stoichiometrically methylated during the initial burst of methylation. The continuing slow production can be explained by slow hydrolysis of the cyclic imide, a reaction that partially regenerates isoACTH (see below).

Isolation and Characterization of the Cyclic Imide Demethylation Product. In an attempt to isolate and characterize the demethylation product, deamidated ACTH was methylated for 90 min under conditions identical with those used in Figure 2A. The resulting ACTH, which was almost completely demethylated, was purified from the reaction mixture by gel filtration on Sephadex G-25 and subjected to cation-exchange HPLC as described under Experimental Procedures. Figure 4 shows that two forms of ACTH were separated on the column. Peak II (71% of the total) exhibited the same retention time, and therefore charge, as native ACTH. Peak I (29% of the total) gave a retention time identical with that of deamidated ACTH, which differs from the native form by one extra negative charge. Peak II results from the quantitative conversion of the isoaspartyl-containing fraction of the deamidated ACTH to the cylcic amide. Peak I is expected

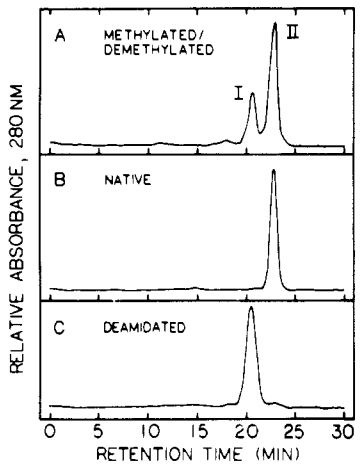

FIGURE 4: Cation-exchange HPLC of deamidated ACTH after one cycle of transient methylation. Deamidated ACTH was incubated in a methylation reaction mixture for 90 min at pH 7.4, 37 °C. It was then isolated from the reaction mixture by gel filtration at pH 4.8 and analyzed by cation-exchange HPLC at pH 6.7 (panel A; see Experimental Procedures for details). Standards of untreated native ACTH (panel B) or untreated deamidated ACTH (panel C) were also chromatographed under identical conditions. Note that peaks I and II have retention times identical with those of deamidated and native ACTH, respectively.

Table II: Methyl-Accepting Capacities of ACTH Peaks I and II from Figure 4[a]

substrate	mol of CH_3/mol of ACTH
deamidated ACTH (control)	0.54
peak I, untreated	0.08
peak II, untreated	0.09
peak II, alkali treated	0.48

[a] Methylation reactions were carried out in duplicate for 40 min at pH 6, 30 °C, in a phosphate–citrate buffer as described previously (Aswad, 1984). Final volumes were 50 μL containing 10 μM ACTH, 2.5 μM PCMT, and 200 μM [*methyl*-^{3}H]AdoMet (400 dpm/pmol). Methyl incorporation was determined from the methanol diffusion assay. Alkaline treatment was for 1 h at pH 9, 37 °C, in 5 mM ammonium hydroxide. The relatively high concentration of PCMT used in these reactions was necessary to ensure maximal methyl incorporation given the low turnover number of the enzyme, 0.27 min^{-1} (Aswad, 1984).

because the residual deamidated ACTH, in which Asp-25 is in a normal peptide linkage, is not a substrate for PCMT.

To further verify the identities of these peaks, we tested their abilities to serve as substrates for PCMT before and after alkaline treatment. As shown in Table II, both peaks are resistant to methylation prior to alkaline treatment. After incubation at pH 9 for 1 h at 37 °C, peak II exhibited a high methyl-accepting capacity. Alkaline treatment is expected to open the cyclic imide ring to produce a ca. 70/30 mixture of isopeptide and normal forms of deamidated ACTH (Murray & Clarke, 1984; Battersby & Robinson, 1955), thus adding further support for the identity of peak II.

Because peak II in Figure 4 exhibits a retention time identical with that of native ACTH, it seemed important to rule out the remote possibility that the demethylation product might actually be native ACTH resulting from a PCMT-catalyzed regeneration of asparagine in position 25. This was done by comparing the rates at which peak II and authentic native ACTH are converted to the more negatively charged forms upon incubation at pH 7.4, 37 °C. The results are shown in Figure 5. As expected, native ACTH exhibits only marginal deamidation under these conditions, even after 16 h of incubation. In contrast, peak II exhibited a $t_{1/2}$ of 4.2 h, a result that clearly rules out its identity as native ACTH

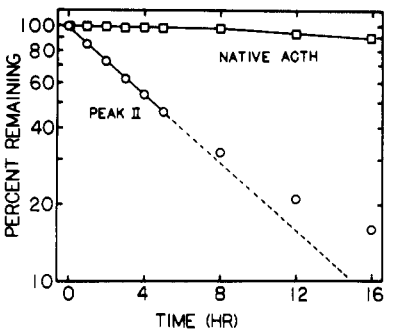

FIGURE 5: Kinetics of conversion of peak II or native ACTH to a more acidic form. Reaction mixtures contained 10 μg of peptide, 50 mM K-HEPES (pH 7.4), and 1 mM EGTA and were carried out at 37 °C in a volume of 1 mL. Reaction mixtures were stopped at the indicated times by freezing at −20 °C and subsequently analyzed by cation-exchange HPLC as in Figure 4. Conversion was measured by integrating the relative peak areas at 20.4 and 22.8 min as in Figure 4. The deviation from linearity observed for peak II is probably due to an error in evaluating the peak II area as it becomes small relative to the 20.4-min peak with which it overlaps.

but that is well within the range expected for a cyclic imide of this type (Bernhard et al., 1962).

The formation of a cyclic imide as the demethylation product can explain the slow second phase of methanol production seen in Figure 2A. The imide slowly regenerates isoACTH with an efficiency of ca. 70%, thereby generating new substrate for PCMT. An endless cycle of methylation–demethylation–imide hydrolysis predicts a limiting value of 3.33 mol of [^{3}H]methanol produced per mole of isoACTH after prolonged incubation.[2] The actual amount of methanol produced in a reaction of limited duration will depend strongly on the pH, more closely approaching the theoretical limit at higher pHs since the rate-limiting step in each cycle is that of imide hydrolysis. We incubated deamidated ACTH for 4 h at 37 °C in reactions buffered at pH 7.0, 8.0, or 9.0 and determined methanol production to be 0.98, 1.75, and 2.83 mol/mol of original isoACTH, respectively.

Does Methyltransferase Influence the Rate of Demethylation-Associated Cyclic Imide Formation? Although imide formation can proceed spontaneously after esterification of the aspartate α-carboxyl in an isoAsp-Gly sequence, we considered the possibility that the methyltransferase might be catalytically influencing the demethylation/imide formation step over and above its role in activating the carboxyl group via esterification. This turned out not to be the case. [^{3}H]-Methyl-labeled isoACTH was prepared enzymatically at pH 6.0 (to minimize demethylation) and purified by reversed-phase HPLC in 0.1 M acetic acid. The demethylation kinetics of 10 μM [^{3}H]methyl-labeled peptide were measured in the presence or absence of 2.5 μM PCMT at 37 °C, pH 7.4. Under these conditions, demethylation was first order with a $t_{1/2}$ of 7.2 min and was not altered by the presence of PCMT.

Overall Mechanism of Methylation/Demethylation of IsoACTH at Physiological pH. On the basis of the results obtained in this study, we can now propose an overall mechanism for the methylation and demethylation of deamidated ACTH under conditions of physiological pH and temperature. This is shown in Figure 3. The isopeptide form of deamidated ACTH is selectively and rapidly methylated in a PCMT-catalyzed reaction. The resulting α-carboxyl methyl ester undergoes rapid and spontaneous demethylation to form a methylation-resistant cyclic imide containing peptide. In a

[2] Calculated for the sum of the infinite geometric series $\sum_{n=1}^{\infty} ar^{n-1} = a/(1 - r)$, where a = 1 mol of isopeptide and r = 0.70, the relative amount of isopeptide re-formed upon hydrolysis of the cyclic imide.

much slower reaction, the imide undergoes spontaneous base-catalyzed ring opening to form a 70/30 mixture of isoAsp/Asp-containing peptides.

All of the kinetic constants that apply to the individual reaction steps in Figure 3 have been measured and are summarized in Table I. These constants have been used to compute the predicted time courses of [*methyl*-^{3}H]isoACTH and [*methyl*-^{3}H]methanol production in Figure 2. The good agreement between the observed and predicted time courses offers substantial evidence for the validity of the proposed scheme.

Discussion

The findings presented here demonstrate that the enzymatic carboxyl methylation of isoACTH at physiological pH is a transient modification that leads to a structural change of considerably longer duration, namely, the formation of a stable cyclic imide between Asp-25 and Gly-26. The transient nature of the methylation reaction is a direct consequence of its unusual substrate specificity. Both the bovine brain (Aswad, 1984) and human erythrocyte (Murray & Clarke, 1984) forms of PCMT selectively methylate the free α-carboxyl group of an atypical isoaspartyl residue, which results from deamidation of a labile asparagine in the sequence Asn-Gly. It is well established that Asn-Gly sequences are prone to deamidation because of the susceptibility of the side-chain carboxyl of asparagine to nucleophilic attack by the α-amino group of glycine (Gräf et al., 1970, 1971). This same mechanism applies to the demethylation of the isoaspartyl methyl ester (Battersby & Robinson, 1955; Bodanszky & Kwei, 1978; Bernhard et al., 1962). Carboxyl methylation might therefore be viewed as an activation step that enhances the reactivity of a specific carboxyl group rather than a final protein modification per se.

The mechanism of methylation and demethylation described here could account for the unusual instability of eucaryotic protein methyl esters. Studies have demonstrated that protein methyl esters formed in vitro by mammalian PCMT from mixtures of cellular proteins (Terwilliger & Clarke, 1981) or purified proteins (Kim & Paik, 1976; Paik & Kim, 1980) are considerably less stable than free aspartate β- or α-methyl esters or chemically formed protein methyl esters. Indeed, attempts to identify endogenous substrates for mammalian PCMT in intact cells or subcellular fractions have generally required the use of special acidic gel electrophoresis systems because the methylated proteins do not survive the alkaline conditions of the more traditional gel systems (Terwillinger & Clarke, 1981; Gagnon et al., 1978; Aswad & Deight, 1983b; MacFarlane, 1983). This contrasts markedly with the γ-glutamyl methyl esters formed on bacterial chemoreceptors, which do survive more alkaline electrophoresis conditions (Kort et al., 1975; Springer & Koshland, 1977).

O'Connor & Clarke (1983) recently characterized membrane-bound protein methyl esters in intact erythrocytes and found that the majority of methylation sites exhibited a $t_{1/2}$ of hydrolysis at pH 8.9, 37 °C, of less than 5 min, a finding that suggests that methylation occurs preferentially on imide-prone aspartyl or isoaspartyl residues. The possibility that in vivo deamidated proteins serve as a major source of natural substrates for mammalian PCMT is also supported by recent findings that the methylation of certain erythrocyte membrane proteins is up to 9-fold greater in the oldest fraction of cells compared with the youngest fraction (Barber & Clarke, 1983; Galletti et al., 1983).

Conversion of an isoaspartyl residue to a stable cyclic imide could provide a mechanism for repair or selective degradation of deamidated proteins. Ammonia could add to the β-carbonyl of the imide, resulting in regeneration of asparagine. Direct addition of ammonia to a cyclic imide can occur under appropriate conditions (Sondheimer & Holley, 1954), but if it occurred in vivo, it would presumably be catalyzed by some as yet uncharacterized enzyme. Alternatively, the imide could react with a primary amino group on another peptide and lead to cross-linking in a reaction analogous to that which occurs when activated ubiquitin is conjugated to a protein and signals its degradation (Hershko & Ciechanover, 1982). A third possibility is that a specific protease recognizes the cyclic imide and cleaves both the α- and β-carboxyl amide bonds. It is of interest to note here that the selective cleavage of Asn–Gly bonds by alkaline hydroxylamine is thought to involve addition of the hydroxylamine to a cyclic imide intermediate (Bornstein & Balian, 1977).

Although the unusual substrate specificity of this enzyme lends itself readily to a possible function in the modification of deamidated proteins, the true intracellular substrates may have a different source. Isoaspartyl residues might occur in certain proteins or peptides by mechanisms other than asparagine deamidation. Alternatively, the true methylation sites might be chemical moieties that resemble but are different from isoaspartyl residues. The methylated (activated) carboxyl group could participate directly in a conjugation reaction such as protein cross-linking or the addition of some other group. If protein carboxyl methylation is viewed as a carboxyl activation step, the true in vivo methyl esters may be even less stable than those that have been found to date. This suggests that a new strategy for identifying the true protein substrates may be appropriate, namely, a search for proteins or protein complexes that support rapid methanol production under physiological conditions.

Acknowledgments

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References

Aswad, D. W. (1984) *J. Biol. Chem. 259*, 10714–10721.

Aswad, D. W., & Deight, E. A. (1983a) *J. Neurochem. 40*, 1718–1726.

Aswad, D. W., & Deight, E. A. (1983b) *J. Neurochem. 41*, 1702–1709.

Axelrod, J., & Daly, J. (1965) *Science (Washington, D.C.) 150*, 892–893.

Barber, J. R., & Clarke, S. (1983) *J. Biol. Chem. 258*, 1189–1196.

Battersby, A. R., & Robinson, J. C. (1955) *J. Chem. Soc.*, 255–265.

Bernhard, S. A., Berger, A., Carter, J. H., Katchalski, E., Sela, M., & Shalitin, Y. (1962) *J. Am. Chem. Soc. 84*, 2421–2426.

Bodanszky, M., & Kwei, J. Z. (1978) *Int. J. Pept. Protein Res. 12*, 69–74.

Bornstein, P., & Balian, G. (1977) *Methods Enzymol. 47*, 132–145.

Bradford, M. E. (1976) *Anal. Biochem. 72*, 248–254.

Chirpich, T. D. (1968) Ph.D. Thesis, University of California, Berkeley.

Clarke, S., & O'Connor, C. M. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 391–394.

Diliberto, E. J., Jr., & Axelrod, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1701–1704.

Diliberto, E. J., Jr., & Axelrod, J. (1976) *J. Neurochem. 26*, 1159–1165.
Edgar, D. H., & Hope, D. B. (1976) *J. Neurochem. 27*, 949–955.
Gagnon, C., & Heisler, S. (1979) *Life Sci. 25*, 993–1000.
Gagnon, C., Viveros, O. H., Diliberto, E. J., Jr., & Axelrod, J. (1978) *J. Biol. Chem. 23*, 3778–3781.
Galletti, P., Ingrosso, D., Nappi, A., Gragnaniello, V., Iolascon, A., & Pinto, L. (1983) *Eur. J. Biochem. 135*, 25–31.
Gear, C. W. (1971) *Numerical Initial Value Problems in Ordinary Differential Equations*, p 35, Prentice-Hall, Englewood Cliffs, NJ.
Gráf, L., Cseh, G., Nagy, I., & Kurcz, M. (1970) *Acta Biochim. Biophys. Acad. Sci. Hung. 5*, 299–303.
Gráf, L., Bajusz, S., Patthy, A., Barát, E., & Cseh, G. (1971) *Acta Biochim. Biophys. Acad. Sci. Hung. 6*, 415–418.
Hershko, A., & Ciechanover, A. (1982) *Annu. Rev. Biochem. 51*, 335–364.
Kim, S., & Paik, W. K. (1971) *Biochemistry 10*, 3141–3145.
Kim, S., & Paik, W. K. (1976) *Experientia 32*, 982–984.
Kim, S., & Li, C. H. (1979) *Int. J. Pept. Protein Res. 13*, 282–286.
Kloog, Y., Flynn, D., Hoffman, A. R., & Axelrod, J. (1980) *Biochem. Biophys. Res. Commun. 97*, 1474–1480.
Kort, E. N., Goy, M. F., Larsen, S. H., & Adler, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3939–3943.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. T. (1957) *J. Biol. Chem. 193*, 265–275.
MacFarlane, D. E. (1983) *Anal. Biochem. 132*, 231–235.
MacFarlane, D. E. (1984) *J. Biol. Chem. 259*, 1357–1362.
Murray, E. D., Jr., & Clarke, S. (1984) *J. Biol. Chem. 259*, 10722–10732.
O'Connor, C. M., & Clarke, S. (1983) *J. Biol. Chem. 258*, 8485–8492.
O'Dea, R. F., Viveros, O. H., & Diliberto, E. J., Jr. (1981) *Biochem. Pharmacol. 30*, 1163–1168.
Paik, W. K., & Kim, S. (1980) *Protein Methylation*, Wiley, New York.
Sondheimer, E., & Holley, R. W. (1954) *J. Am. Chem. Soc. 76*, 2467–2470.
Springer, W. R., & Koshland, D. E., Jr. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 533–537.
Terwilliger, T. C., & Clarke, S. (1981) *J. Biol. Chem. 256*, 3067–3076.
Trivedi, L, Gupta, A., Paik, W. K., & Kim, S. (1982) *Eur. J. Biochem. 128*, 349–354.
Wold, F. (1981) *Annu. Rev. Biochem. 50*, 783–814.