Carboxyl Methylation of Deamidated Calmodulin Increases Its Stability in *Xenopus* Oocyte Cytoplasm

**IMPLICATIONS FOR PROTEIN REPAIR***

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The widely distributed protein-L-isoaspartate(0-aspartate) O-methyltransferase (PIMT; EC 2.1.1.77) is postulated to play a role in the repair or metabolism of damaged cellular proteins containing L-isoaspartyl residues derived primarily from the spontaneous deamidation of protein asparaginyl residues. To evaluate the functional consequence of PIMT-catalyzed methylation on the stability of isoaspartyl-containing proteins in cells, *Xenopus laevis* oocytes were microinjected with both deamidated and nondeamidated forms of recombinant chicken calmodulin (CaM) containing a hemagglutinin (HA) epitope at its N terminus. Processing of HA-CaM was monitored by electrophoretic analysis and Western blotting of oocyte extracts. The experiments indicate that deamidated HA-CaM is degraded after microinjection, while nondeamidated HA-CaM is stable. Kinetic analysis is consistent with the entry of microinjected HA-CaM into two intracellular pools with distinct hydrolytic stabilities. The larger, more stable pool may consist of HA-CaM bound to the heterogeneous pool of oocyte CaM binding proteins detected by an overlay procedure. Enzymatic methylation of deamidated HA-CaM with purified PIMT prior to injection results in its stabilization. Conversely, inhibition of endogenous oocyte PIMT with sinuefungin, a nonhydrolyzable analog of S-adenosylhomocysteine, increases the rate of deamidated HA-CaM degradation. These results are consistent with a role for PIMT-catalyzed methylation in the repair of damaged cellular proteins.

All living cells contain enzymatic systems that maintain a functional pool of cellular proteins by catalyzing the refolding, repair, or removal of structurally damaged proteins (1, 2). In some cases, these enzymes recognize modified or abnormal amino acid residues that have arisen spontaneously during the aging of the protein (3, 4). One such activity is a protein L-isoaspartate(0-aspartate) O-methyltransferase (PIMT; EC 2.1.1.77) that appears to be of ancient origin, with homologues identified in bacterial, plant, and animal cells (5, 6). All of the widely distributed PIMT activities specifically recognize L-isoaspartyl residues that can spontaneously arise from either the deamidation of protein asparaginyl residues or the isomerization of protein aspartyl residues (7, 8). It has been demonstrated for several proteins, including epidermal growth factor, calmodulin (CaM), and calbindin (9–11), that the appearance of an isoaspartyl site is correlated with a loss of normal enzyme function. It has been proposed that PIMT-catalyzed carboxyl methylation of isoaspartyl sites is the first step in either the repair or degradation of the damaged protein substrate (3, 9, 12).

Evidence supporting a repair function has come from studies of PIMT-catalyzed methylation in vitro. In several experiments, isoaspartyl-containing synthetic peptides were nearly stoichiometrically converted to the corresponding aspartyl-containing form following carboxyl methylation by PIMT and the ensuing internal rearrangements associated with hydrolysis of the ester, involving a succinimide intermediate (13, 14). The repair process, however, lacked the strict stereochemical specificity and efficiency characteristic of enzymatic systems, requiring multiple rounds of methylation and demethylation to achieve full repair. Experimental evidence has also been presented implicating PIMT in the repair of damaged protein substrates. When deamidated, isoaspartyl-containing forms of the bacterial HPr phosphocarrier protein (12) or calmodulin (9) were used as substrates for the PIMT, carboxyl methylation partially restored enzymatic activity that had been lost as a consequence of the original deamidation. Significantly, however, full enzymatic activity was not recovered, and isoaspartyl residues were not replaced by the original asparaginyl residues.

At the present time, there is no evidence supporting the other proposed role for the PIMT in marking damaged substrates for degradation, although proteolytic destruction of damaged proteins would also serve to maintain a functional pool of cellular proteins. Based on the available evidence, which is all derived from experiments using purified systems (9, 13, 14), it would be premature to exclude a physiological role for the PIMT in the destruction of damaged isoaspartyl-containing proteins. Purified systems may lack hitherto unidentified activities that participate in the processing of isoaspartyl-containing proteins within cells.

From these studies, it has become clear that elucidation of the biochemical pathway initiated by carboxyl methylation would be assisted by the development of a model for studying the processing of methylated proteins in intact cells. We have chosen *Xenopus* oocytes for a model because these cells possess a functional excess of PIMT similar to those activities previously studied in more detail in mammalian systems (15, 16).
and because these large cells are easily injected with macromolecules. For the present studies, we constructed an epitope-tagged CaM (17) as a model substrate, enabling us to follow its fate after microinjection into oocytes. CaM was chosen for several reasons. CaM acts as a physiological methyl acceptor in several cell types, including both the human erythrocyte (18, 19) and the Xenopus oocyte (20). Careful measurement of the methylation stoichiometry in the two cells has revealed that about 0.02–0.03% of the CaM is carboxyl methylated at steady state, reflecting the low abundance of isoaspartyl residues generally in proteins. Peptide mapping of erythrocyte CaM (21) indicated that physiological methylation occurs at isoaspartyl residues in the central helix and calcium-binding sites as well as near the N terminus. Prolonged incubation of native CaM at the near physiological conditions of pH 7.4 and 37 °C for 2 weeks in vitro produces isoaspartyl residues at these same sites (22, 23), indicating that it is possible to approximate the naturally occurring age-related changes in CaM in the test tube.

We have used the same protocols to produce isomerized variants of an epitope-tagged CaM for microinjection into oocytes. The presence of a hemagglutinin (HA) epitope is advantageous in allowing the injected substrate to be detected by immunological methods (17).

This experimental design has allowed us to evaluate potential roles of PIMT-catalyzed methylation in cellular protein metabolism. Our results show that the presence of isoaspartyl residues severely compromises the stability of CaM in oocyte cytoplasm. PIMT-catalyzed methylation of damaged HA-CaM prior to microinjection increases its stability, while inhibition of the endogenous PIMT activity decreases the stability of injected HA-CaM. These results are most consistent with the involvement of PIMT in the structural repair of damaged proteins in cells and do not support a role for PIMT in targeting damaged proteins for proteolysis.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant HA-CaM and Deamidated HA-CaM Variants—Epitope-tagged CaM containing the HA epitope (YPDYDVPDYA) at its N terminus (17) was prepared from E. coli N-4830 cells containing a temperature-sensitive cl repressor protein. To induce recombinant HA-CaM synthesis, bacterial cultures were grown overnight at the permissive temperature of 30 °C, and the cultures were then diluted to an A600 of 0.4–0.5 with medium prewarmed to the nonpermissive temperature of 41 °C to induce HA-CaM synthesis. After growth at 41 °C for 2 h, bacteria were pelleted by centrifugation for 5 min at 8000 × g. The cell pellet was resuspended in 0.02 volumes of ice-cold lysis buffer containing 10 mM Tris, 1 mM EDTA, 5 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.25 mg/ml lysozyme. HA-CaM was purified from the cell lysate through a nickel-chelate affinity column equilibrated in 50 mM Tris, pH 7.4, 5 mM CaCl2, 500 mM NaCl. The column was washed with 5 volumes of starting buffer, after which HA-CaM was eluted with the buffer containing 1 mM EDTA. Deamidated variants of HA-CaM were prepared by incubating solutions of 100 μM HA-CaM in 50 mM HEPES, 1 mM EDTA, pH 7.4, at 37 °C for 2 weeks (9). Control HA-CaM was incubated in the same buffer containing 1 mM CaCl2 in the place of EDTA.

Enzymatic Carboxyl Methylation of CaM—The acidic isozyme of PIMT was purified from calf brain as described (30). To measure methyl-accepting activities, CaM and HA-CaM samples were incubated with 22 mM PIMT, 10 μM S-adenosyl-L-[methyl-3H]methionine (adjusted to a final specific activity of 1 Ci/mmol with nonradioactive AdoMet) in 25 mM MES, pH 6.5, for 2 h at 37 °C in a final volume of 25 μl. Protein carbonyl [3H]methyl esters were quantified as trichloroacetic acid-precipitable material converted to [3H]methanol by base treatment, as described previously (16). Each assay was done in triplicate and included a negative control lacking substrate, representing the assay background. Carbonyl methylated HA-CaM was prepared for microinjection using the same procedures, except that radioactivity was omitted from the mixture.

RESULTS

The HA Epitope Does Not Affect the Spontaneous Deamidation of CaM—Previous experiments have shown that the addition of the HA epitope to the N terminus of CaM has no effect on the processing of the protein. We have further shown that HA-CaM cannot be deamidated in vitro. Thus we have determined the specific activities of the purified PIMT to test whether the addition of the HA epitope affects the processing of CaM in vivo. 100 nM purified CaM and HA-CaM were incubated with 22 mM PIMT and S-adenosyl-L-[methyl-3H]methionine (2 Ci/mmol) for 2 or 4 h at 37 °C in a final volume of 25 μl. The samples were then subjected to a standard SDS-PAGE system containing 2 mM EDTA as described (9). Denaturing SDS-PAGE on 15% gels was carried according to the protocol described by Laemmli (31). HA-CaM bands from the gel were de-
on the enzymatic activity of CaM, suggesting that HA-CaM could be used to model CaM functions (17). We have been interested in using CaM as a model substrate for PIMT because of the ease with which CaM can be converted into isoaspartyl-containing variants under the physiologically relevant conditions of a 2-week incubation at pH 7.4 and 37 °C in the presence of EGTA (9, 22, 31). Following such incubations, one detects new electrophoretic variants that migrate more slowly than native CaM due to the spontaneous generation of methyl-accepting isoaspartyl residues at several sites in the protein, including the calcium-binding sites (9, 20).

In order for HA-CaM to effectively serve as a model for carboxyl methylation, it was necessary to demonstrate that the HA epitope did not adversely affect the spontaneous deamidation of CaM to these methyl-accepting forms. In the experiment shown in Fig. 1, we have used the same nondenaturing gel system to characterize CaM and HA-CaM variants produced during incubation of the samples for 14 days at pH 7.4 and 37 °C in the presence of either 1 mM CaCl₂ (lanes 1 and 3) or 1 mM EDTA (lanes 2 and 4). In both cases, incubation in the presence of EDTA generates electrophoretically distinct forms that migrate more slowly than the native forms. Essentially identical results are obtained with the CaM and HA-CaM samples, except that the HA-tagged forms migrate more slowly than their untagged counterparts. All of the variants generated by prolonged incubation of HA-CaM are readily detected using a monoclonal antibody to the HA epitope on immunoblots (Fig. 1, right panel).

Analysis of CaM and HA-CaM Tryptic Fragments by MALDI-TOF Mass Spectrometry—In order for “aged” HA-CaM (Fig. 1) to serve as a model substrate for PIMT-dependent metabolism in cells, it was important to establish that the 2-week incubation did not produce changes in amino acids other than isoaspartyl generation. It was also important to eliminate the possibility that some of the new electrophoretic variants detected in Fig. 1 represented proteolytic fragments of CaM or HA-CaM, particularly in light of the heightened susceptibility of CaM to proteolytic cleavage in the presence of EGTA (32, 33). Tryptic peptides were therefore prepared from the major variant in each sample in Fig. 1, and the fragments were analyzed using MALDI-TOF mass spectrometry. The mass spectrum for the peptide mixture obtained from native CaM identifies that protein as authentic CaM. All of the masses detected in Fig. 2A correspond to the singly charged ions of known tryptic fragments, as indicated. In addition, two peptides, corresponding to residues 127–148 (MH⁺ = 2493) and residues 38–74 (MH⁺ = 4071), are associated with oxidized methionyl variants 16 or 32 mass units larger than the parent peptide, in good correspondence to the number of methionyl residues in the peptide. These oxidized methionyl variants are characterized during the MALDI-TOF analyses. The addition of the HA epitope to CaM produces a set of tryptic fragments (Fig. 2B) consistent with the presence of the sequence AYPYDVDPYAM that was added to the N terminus of CaM during the construction of HA-CaM (17). The ions MH⁺ = 1598 and MH⁺ = 3822 (indicated with asterisks in Fig. 2, C and D), corresponding to residues 78–90 and 116–148 of CaM, are detected in both deamidated CaM (Fig. 2A) and deamidated HA-CaM (Fig. 2D). These fragments are consistent with a lack of cleavage after Arg¹²⁴ and Arg¹²⁶, suggesting that deamidation reduces susceptibility to tryptic cleavage at these two sites. Overall, the incubation used to produce deamidated CaM does not alter the masses of any identified ions, suggesting that changes are limited to aspartyl isomerization and asparaginyl deamidation (21, 34, 35). Unfortunately, the resolution of MALDI-TOF analysis is not sufficiently sensitive to confirm the change of 1 mass unit that would be expected from the conversion of an asparaginyl residue to an aspartyl residue. Significantly, ions representing the intact N-terminal and C-terminal peptides of CaM can be identified in all samples, confirming that proteolytic digestion is not responsible for the altered electrophoretic variants generated during the 2-week incubation of CaM used to produce deamidated CaM. This is particularly important in light of a previous report that a brain carboxypeptidase produces a shortened CaM with enhanced methyl-accepting activity (36).

Methyl-accepting Activity of Deamidated HA-CaM—Because of its unique specificity for protein d-aspartyl and l-isoaspartyl residues, the PIMT has proven to be a useful tool for detecting isoaspartate in peptides and proteins (37, 38). To determine if the isoaspartyl content of HA-CaM is increased by prolonged incubation in the absence of calcium, we compared the specific methyl-accepting activities of the CaM and HA-CaM produced during a 2-week incubation at 37 °C in either the absence or presence of calcium. Methyl-accepting activities were assessed following a 2-h incubation of the samples with a high concentration of PIMT and 10 μM [³H]AdoMet. As shown in Table I, CaM and HA-CaM samples demonstrate very low methyl-accepting activities following an incubation in the presence of calcium, confirming earlier reports that isoaspartyl residues are not readily formed in the more highly structured, calcium-ligated CaM. By contrast, both HA-CaM and CaM display high methyl-accepting activity following incubation in the absence of calcium. The increase in methyl-accepting activity observed in the absence of calcium is even greater for HA-CaM than for CaM, indicating that the HA epitope does not interfere with the production of l-isoaspartyl residues. Under the same experimental conditions, ovalbumin, a commonly used substrate for the PIMT, shows significantly lower methyl-accept-
ing activity, which is nonetheless consistent with literature values for its specific methyl-accepting activity (16).

**Stability of Native and Deamidated HA-CaM in Oocytes**—Previous studies have shown that a small fraction of the endogenous CaM in oocytes is methylated by the PIMT in vivo, indicating that deamidation of CaM occurs in the oocyte (20). It is not possible to determine from existing data, however, if deamidation and carboxyl methylation affect the stability of oocyte CaM. To determine if deamidation affects CaM stability, oocytes were microinjected with approximately 100 ng of either native or deamidated HA-CaM. The quantity of deamidated HA-CaM injected was 3–4 times that required for half-maximal stimulation of the endogenous oocyte PIMT activity (20). At various times after injection, the cytosolic fraction was prepared from pools of five oocytes, and the quantity of HA-CaM remaining was determined by using the quantitative immunoblot procedure (17) described under “Experimental Procedures” (Fig. 3). Samples were analyzed by both SDS-PAGE (Fig. 3) and by nondenaturing gel electrophoresis (Fig. 4) to determine the total HA-CaM remaining after injection as well as the distribution between electrophoretic variants, respectively. In all cases, a single band was detected in samples resolved by SDS-PAGE, and these data have been expressed quantitatively in Fig. 3.

As shown in Fig. 3, native HA-CaM is stable in oocytes for at least 20 h. Interestingly, recombinant HA-CaM lacks methyl groups at Lys-115, a potential site of monoubiquitination in model systems (39, 40). This surface-exposed Lys-115 (41) is normally methylated in Xenopus CaM (42) and is rapidly methylated when recombinant CaM is injected into oocytes (20). We do not observe any increase in the apparent molecular weight of injected CaM consistent with ubiquitination, and it is clear from Fig. 3 that little, if any, of the microinjected nonincubated

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**Table I**

| Protein substrate      | Specific methyl-accepting activity (pmol esters/nmol protein) |
|------------------------|---------------------------------------------------------------|
| Native calmodulin      | 2.70 ± 0.02                                                   |
| Deamidated calmodulin  | 181 ± 10.4                                                    |
| Native HA-calmodulin   | 1.40 ± 0.10                                                   |
| Deamidated HA-calmodulin | 276 ± 21.3                                                  |
| Ovalbumin              | 2.60 ± 0.30                                                   |

**Fig. 2.** Tryptic peptide analysis of recombinant CaM and recombinant HA-CaM by MALDI-TOF mass spectrometry. Samples of recombinant CaM (A and C) and recombinant HA-CaM (B and D), were incubated at pH 7.4 and 37 °C for 14 days in the presence of either 1 mM CaCl₂ (A and B) or EDTA (C and D) to generate the electrophoretic variants shown in Fig. 1. Following the incubation, samples (0.1 μg) were resolved by nondenaturing PAGE as in Fig. 1, and proteins were transferred to a polyvinylidene difluoride membrane. Proteins were visualized with Amido Black staining, and the membrane segments containing the major electrophoretic variant in each lane was excised and used for tryptic peptide analysis as described under “Experimental Procedures.” Tryptic fragments were analyzed by MALDI mass spectrometry. Individual peptides were identified by their mass as indicated. The asterisks indicate new peptide fragments generated in samples incubated in the presence of EDTA.

**Fig. 3.** Decreased stability of deamidated recombinant HA-CaM in oocytes. Xenopus oocytes were microinjected with 46 nl of solution containing 114 ng of native or deamidated HA-CaM and incubated in groups of five for varying periods of time at 20 °C. Cytosolic fractions were prepared as described under “Experimental Procedures” and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and HA-tagged proteins were detected by Western blotting. Results were quantified by comparison with standard lanes containing 40, 80, and 120 ng of recombinant HA-CaM. A single immunoreactive species was detected in each lane by Western blotting. Data were quantified by scanning densitometry, and the results are reported as the percentage of remaining native (●) or deamidated (■) recombinant HA-CaM, respectively. Each point represents the average of three determinations with the S.D.
HA-CaM is targeted for proteolytic degradation under our experimental conditions.

In sharp contrast, isoaspartyl-containing HA-CaM is actively degraded following microinjection into oocytes with biphasic kinetics. Less than half of the injected isomerized HA-CaM is present 5 h after injection, and only about 20% remains after 20 h. Because the quantity of injected material is in excess of that required to saturate the oocyte PIMT (20), most of the proteolytic processing observed should be independent of PIMT activity. It is not possible to determine from these data if the small amount of injected HA-CaM remaining after 20 h has been methylated by the PIMT. The results of Fig. 3 clearly establish, however, the existence of a metabolic pathway not involving the PIMT that selectively distinguishes isomerized from native HA-CaM and initiates its proteolytic destruction. From the results of the nondenaturing gel in Fig. 4, it appears that all of the isoelectric variants generated during the extended incubation used to produce deamidated CaM are degraded with similar kinetics.

Concentration Dependence of HA-CaM Metabolism—In the experiment of Fig. 5, the concentration dependence of isomerized HA-CaM metabolism in oocytes was evaluated by injecting oocytes with various concentrations of HA-CaM and observing the kinetics of its disappearance from the cytoplasm. The quantities of HA-CaM injected in these experiments, determined immediately after injection, ranged from 5 to 114 ng, as indicated in the figure legend. Quite distinct kinetic patterns were observed at low and high concentrations of injected HA-CaM. At the highest concentrations of 50 and 114 ng injected, HA-CaM is proteolyzed with monophasic kinetics characterized by a half-time of approximately 5 h. At the lowest concentrations of 5 ng, the microinjected HA-CaM is hydrolyzed much more rapidly and largely disappears within 3 h after injection. More complex kinetics, consistent with two kinetic compartments, are observed at the intermediate concentration of 25 ng of injected HA-CaM.

A similar concentration dependence is not observed when native, nonisomerized HA-CaM is injected into oocytes (data not shown). Although native and isomerized HA-CaM very likely enter some of the same intracellular compartments (see below), the native HA-CaM is protected from degradation in both compartments.

Multiple CaM-binding Proteins Detected in Oocytes—In most cell types, CaM interacts with a large number of proteins. Similarly, it is possible that the association of isomerized HA-CaM with CaM-binding proteins in oocytes could confer protection from the proteolytic system that recognizes isomerized variants of HA-CaM. In this respect, it has been reported that the association of CaM with phosphorylase protects CaM from ubiquitination in a model system (43). In the experiment of Fig. 6, we have used an overlay method (17) to identify proteins in oocyte cytosol that specifically interact with HA-CaM and deamidated HA-CaM. As shown in Fig. 6, both native and deamidated HA-CaM bind to multiple proteins in oocyte extracts in a calcium-dependent manner. Prominent HA-CaM binding proteins with calculated sizes of 48, 54, 60, 68, 85, and 91 kDa interact with HA-CaM in the presence, but not the absence, of calcium. The pattern of binding proteins observed using deamidated HA-CaM is virtually identical to that observed with native HA-CaM, suggesting that deamidation has very little effect on the ability of HA-CaM to interact with oocyte proteins. The only exception is an interaction of deamidated HA-CaM with a 48-kDa protein, which is only partially reduced by the removal of calcium. Although the actual binding proteins have not been identified, the large number of HA-CaM-binding proteins detected in oocytes could constitute a compartment for sequestering injected deamidated HA-CaM and delaying its degradation.

Carboxyl Methylolation of Isomerized HA-CaM Retards Its Degradation in Oocytes—The metabolic fate of proteins methylated by the PIMT is unknown. Based on the specificity of the enzyme for isoaspartyl-containing proteins, it has been proposed that PIMT initiates either the repair or degradation of its substrates (3, 4). These two potential roles for PIMT in protein metabolism predict opposite effects on the stability of the substrate proteins following methylation. As a first step in discriminating between these two potential roles, we have evaluated the effect of carboxyl methylation on the stability of deamidated HA-CaM in oocytes. To ensure that all of the injected HA-CaM entered the smaller, more metabolically active pool of CaM in oocytes (Fig. 5), groups of oocytes were microinjected with 1.7 ng of deamidated HA-CaM. One group of oocytes was injected with HA-CaM that had been preincubated with the PIMT and AdoMet under the conditions described in Table I prior to injection. The fractional methylation in this preparation was estimated as 0.28 pmol methyl esters/pmol protein, based on the quantity of radioactivity incorporated into HA-CaM methyl esters in a parallel sample supplemented with [3H]AdoMet. A control group was injected with HA-CaM that was preincubated with AdoMet in the absence of PIMT for a similar period of time.

It is apparent from the data of Fig. 7 that prior carboxyl methylation by the PIMT significantly stabilizes deamidated HA-CaM in oocyte cytoplasm. At 30 min after injection, approximately 45% of the nonmethylated HA-CaM has been de-
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graded, in contrast to the 20% of the methylated sample. At 2 h after injection, 66% of the nonpremethylated HA-CaM has been degraded, in contrast to 34% of the premethylated samples. The difference between the two values corresponds well to the fractional methylation of HA-CaM molecules.

Inhibition of the Oocyte PIMT Increases the Hydrolysis of Deamidated Recombinant HA-CaM in Oocytes—Previous experiments have shown that the endogenous oocyte PIMT specifically recognizes and methylates deamidated variants of brain CaM microinjected into oocytes (20), although stoichiometric methylation of the injected deamidated CaM was not observed. From the foregoing results, it appears that the lack of full methylation observed in this previous study (20) can be explained by hydrolytic destruction of the injected substrate prior to encountering the oocyte PIMT. One would predict from the results of Fig. 7, however, that the fraction of deamidated HA-CaM that is methylated by the endogenous oocyte PIMT following microinjection should be protected from proteolysis. In the experiment of Fig. 8, we have tested this possibility directly by comparing the rate of deamidated HA-CaM destruction in untreated oocytes with the corresponding rate in oocytes pretreated with sinefungin (44), an S-adenosylhomocysteine analog that has been previously shown (28) to effectively inhibit the endogenous oocyte PIMT. In this experiment, oocytes were injected with sinefungin or buffer approximately 30 min prior to the injection of 5 ng of deamidated HA-CaM. The presence of sinefungin in oocyte cytoplasm results in an accelerated rate of deamidated HA-CaM hydrolysis (Fig. 8), consistent with a role for protein carboxyl methylation in preventing hydrolysis of methylated proteins. Comparing Figs. 7 and 8, it is clear that premethylation of the deamidated HA-CaM with purified PIMT conferred significantly greater stabilization of the substrate than could be achieved with the endogenous PIMT, reflecting the higher fractional methylation of HA-CaM molecules.

**DISCUSSION**

The wide distribution of PIMT activity and its strong conservation through evolution (6) suggest that the metabolism of protein isoaspartyl residues is an essential cellular function. Based on the unusual specificity of the PIMT for age-damaged aspartyl residues, it has been proposed that the PIMT prevents the accumulation of dysfunctional isoaspartyl-containing proteins in cells by initiating either their repair or degradation (3, 4). Direct evidence for either role has been lacking, however, because protein [3H]methyl esters formed during the incubation of intact cells with [1-3H]methionine are rapidly hydrolyzed, preventing one from following the fate of methylated proteins in vivo. To overcome these limitations, we have developed a microinjection model for following the fate of PIMT substrates after their methylation (16, 20). The model substrate used for this study is an epitope-tagged CaM that is readily converted to an isoaspartyl-containing form by an extended incubation under relatively physiological conditions of temperature and pH. The results of the microinjection study described here indicate that oocytes possess a proteolytic pathway that selectively degrades isoaspartyl variants of HA-CaM and that carboxyl methylation of isoaspartyl-containing HA-CaM protects the molecule from degradation.

The presence of an HA epitope at the N terminus of CaM allows one to follow the fate of the microinjected HA-CaM. As described previously, the addition of the HA epitope to CaM...
has no discernible effect on its enzymatic activity (17), and we show here that it also does not affect its conversion to an isoaspartyl, methyl-accepting form. Thus, it is possible to follow the metabolism of both native and deamidated CaM in *Xenopus* oocyte cytoplasm. As shown in Fig. 3, native HA-CaM is very stable in oocyte cytoplasm, with little or no degradation observed over a 19-h incubation period. This result suggests that the injected HA-CaM equilibrates with the pool of endogenous CaM after injection, which was predicted from a previous biosynthetic labeling study to turn over with a half-time of several weeks (45).

Deamidated HA-CaM, by contrast, is markedly unstable following microinjection into oocytes and is largely degraded within 19 h after injection (Fig. 3). Similar results were obtained when samples were analyzed using SDS-PAGE or using a nondenaturing gel system that resolves several new electrophoretic variants generated by deamidation of HA-CaM. Individual electrophoretic variants have not been structurally characterized, but the variants are expected to represent a complex mixture of singly and multiply isomerized forms, because of the ability of isoaspartyl sites to form at numerous positions in the CaM sequence (22, 23). As shown in Fig. 4, individual variants were hydrolyzed with similar kinetics, suggesting that the proteolytic mechanism recognizes a variety of isoaspartyl-containing forms.

The quantities of deamidated HA-CaM injected into oocytes in the experiments of Figs. 3 and 4 are roughly similar to the total content of endogenous CaM (45) and exceed the amount previously shown to saturate the oocyte PIMT in microinjection studies (20). When much smaller quantities of isomerized HA-CaM are microinjected, more rapid degradation is observed (Fig. 5). The kinetics of isomerized HA-CaM degradation in the oocytes show an inverse correlation with the concentration of injected HA-CaM (Fig. 5), suggesting that injected HA-CaM equilibrates with at least two compartments within oocytes characterized by widely different susceptibility to proteolytic digestion. The simplest interpretation of these data is that a small amount of injected HA-CaM enters a metabolically active pool susceptible to rapid proteolytic degradation and that this pool is saturated at very low concentrations of injected HA-CaM. The major portion of the injected HA-CaM enters a second larger and much more stable pool, disappearing with a half-life of about 5 h. The increase in half-life noted with increasing concentrations of injected HA-CaM could reflect transfer of deamidated HA-CaM from the larger stable pool into the smaller unstable pool.

Although the physical nature of the CaM pools in oocytes is unknown, the smaller pool could well represent uncomplexed CaM, and the larger pool could represent CaM complexed to other oocyte proteins. The overlay data in Fig. 6 demonstrate that HA-CaM is capable of associating with a diverse group of oocyte proteins. The identities of the CaM-binding proteins have not been established, but logical candidates include an 86-kDa membrane skeleton protein and the 50-kDa neuro-modulin (GAP-43), both of which have been identified in *Xenopus* oocyte extracts (46, 47). In addition, an enzymatic activity consistent with a CaM-dependent protein kinase II (47) has been identified in oocyte extracts, and a calcineurin cDNA has been cloned from oocytes (GenBank™ accession no. 2827899). The association of injected HA-CaM with oocyte CaM-binding proteins may protect deamidated HA-CaM from proteolytic degradation. In this respect, it has been shown that CaM is not polyubiquitinated in cell-free systems when complexed to phosphorylase kinase but can be polyubiquitinated and directed to the proteosome when not associated with CaM-binding proteins (43). Currently, the nature of the proteolytic system responsible for deamidated HA-CaM degradation in oocytes is not clear and might involve the ubiquitin/proteosome pathway.

The data presented here support the proposal that structural alterations in deamidated proteins increase their susceptibility to proteolytic action, a hypothesis based on the observed inverse relation between the amide content and the half-life of proteins (48). Our results suggest that isoaspartyl residues derived from deamidation may either directly or indirectly, by initiating other structural changes in the protein structure, act as a recognition signal for a proteolytic system in oocytes. Similarly, Artigues *et al.* (49) have shown that a deamidated variant of serine hydroxymethyltransferase activity is unstable when microinjected into oocytes and that the stability of the protein is increased when a N-terminal fragment containing the isoaspartyl residue is removed by prior chymotrypsin digestion. Unfortunately, however, the rate of degradation was not measured for native serine hydroxymethyltransferase, so it was not possible to determine if the presence of L-isoaspartyl residue was responsible for targeting to the proteolytic machinery. Our data comparing the stability of native and deamidated HA-CaM (Fig. 3) suggest that the presence of the L-isoaspartyl residues is sufficient to mark damaged proteins for proteolytic destruction.

Our microinjection model has allowed us to evaluate the role of PIMT-catalyzed methylation on the metabolism of isoaspartyl-containing proteins. Previous proposals that PIMT initiates either the repair or degradation of its damaged substrate proteins lead to quite different predictions for the impact of carboxyl methylation on protein stability (3, 4). We have used two approaches to assess the effect of carboxyl methylation on the stability of deamidated recombinant HA-CaM in oocytes. In the experiment of Fig. 7, we have compared the stabilities of both methylated and nonmethylated deamidated HA-CaM following microinjection into oocytes. To ensure a high methylation stoichiometry, the HA-CaM was premethylated with purified bovine brain PIMT prior to microinjection. These results of this experiment indicate that carboxyl methylation significantly increases the stability of deamidated CaM. Interestingly, the fractional methylation, estimated as 28% in this experiment (Table I), is very similar to the fraction of injected HA-CaM protected from degradation. It is not possible in this experiment to determine how carboxyl methylation by the endogenous PIMT contributes to the results, although the endogenous enzyme would be expected to contribute equally to both groups of oocytes monitored in Fig. 7. To evaluate the effect of the endogenous PIMT on recombinant HA-CaM stability in oocytes, we have measured the effect of sinefungin, an S-adenosylhomocysteine analog (44), on the hydrolysis of injected substrate (Fig. 8). In good agreement with the previous results, the presence of sinefungin results in an accelerated rate of recombinant HA-CaM degradation. Thus, the enzymatic carboxyl methylation of deamidated recombinant HA-CaM by either heterologous or endogenous PIMT has a stabilizing effect on the substrate. Both sets of results are more consistent with a role for PIMT in repairing structural damage to the protein than in initiating degradation of the damaged substrate.

From the results of previous biosynthetic labeling experiments, it is likely that CaM carboxyl methylation is physiologically significant in oocytes. These experiments have established that CaM is a very stable protein in oocytes (45) and that only about 0.02% of the endogenous oocyte CaM is carboxyl-methylated (20), reflecting a low representation of isoaspartyl sequences. The maintenance of a low isoaspartyl content can be attributed to the efficient metabolism of damaged CaM by a PIMT-initiated pathway, the degradative pathway described here, or a combination of the two. Our results have revealed an
interplay between carboxyl methylation and proteolysis in cells. From these results, we speculate that carboxyl methylation may be the first line of defense against the accumulation of deamidated proteins, possibly initiating the structural repair of the damaged protein. Cells may then use proteolytic mechanisms to remove damaged proteins that have not been methylated, thereby maintaining a functional pool of cellular proteins.

REFERENCES

1. Stadtman, E. R. (1992) Science 257, 1220–1224
2. Gehring, M. J., and Sambrook, J. (1992) Nature 355, 33–45
3. Clarke, S. (1985) Annu. Rev. Biochem. 54, 479–506
4. Aswad, D. W. (1989) Curr. Opin. Cell Biol. 1, 1182–1187
5. Johnson, B. A., Ngo, S. Q., and Aswad, D. W. (1991) Biochem. Int. 24, 841–847
6. Kagan, R. M., McFadden, H. J., McFadden, P. N., O’Connor, C., and Clarke, S. (1997) Comp. Biochem. Physiol. 117B, 379–385
7. Geiger, T., and Clarke, S. (1987) J. Biol. Chem. 262, 785–794
8. Stiegher, R. C., and Clarke, S. (1991) J. Biol. Chem. 264, 6164–6170
9. Johnson, B. A., Langmack, E. L., and Aswad, D. W. (1987) J. Biol. Chem. 262, 12283–12287
10. George-Nascimento, C., Lowenson, J., Borissenko, M., Calderón, M., Medina-Selby, A., Kuo, J., Clarke, S., and Randolph, A. (1990) Biochemistry 29, 9584–9591
11. Chazin, W. J., Koerdel, J., Thulin, E., Hofmann, T., Drakenberg, T., and Forse’n, T. (1987) FEBS Lett. 21368–21374
12. Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B., and Clarke, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5622–5629
13. McFadden, P. N., and Clarke, S. (1986) FEBS Lett. 269, 276–286
14. Johnson, B. A., Shirokawa, J. M., Hancock, W. S., Spellman, M. W., Basa, L. J., and Aswad, D. W. (1989) J. Biol. Chem. 264, 14262–14271
15. Ota, I. M., and Clarke, S. (1987) J. Biol. Chem. 262, 5469–5475
16. Romanik, E. A., and O’Connor, C. M. (1987) J. Biol. Chem. 262, 16404–16411
17. Szymanska, G., O’Connor, M. B., and O’Connor, C. M. (1997) Anal. Biochem. 252, 96–103
18. Brunauer, I., and Clarke, S. (1986) Biochem. J. 236, 811–820
19. Runte, L., Jürgensmeier, C. U., and Seling, H. D. (1982) FEBS Lett. 147, 125–130
20. Desrosiers, R. R., Romanik, E. A., and O’Connor, C. M. (1990) J. Biol. Chem. 265, 21368–21374
21. Ota, I. M., and Clarke, S. (1990) Arch. Biochem. Biophys. 270, 320–327
22. Ota, I. M., and Clarke, S. (1989) Biochemistry 28, 4020–4027
23. Potter, S. M., Henzel, W. J., and Aswad, D. W. (1993) Protein Sci. 2, 1648–1663
24. Putkey, J. A., Slaughter, G. R., and Means, A. R. (1985) J. Biol. Chem. 260, 4704–4712
25. Laemmli, U. K., and Favre, M. (1973) J. Mol. Biol. 80, 575–599
26. Slaughter, G. R., and Means, A. R. (1987) Methods Enzymol. 139, 133–144
27. Wallace, R. A., Jared, D. W., Dumont, J. N., and Segal, M. W. (1973) J. Exp. Zool. 184, 321–334
28. O’Connor, C. M., and Meriman, B. J. (1987) J. Biol. Chem. 262, 10404–10411
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Aswad, D. W., and Deight, E. A. (1983) J. Neurochem. 41, 1702–1709
31. Johnson, B. A., Shirokawa, J. M., and Aswad, D. W. (1989) Arch. Biochem. Biophys. 268, 276–286
32. Klee, C. B., and Vanaman, T. C. (1982) Adv. Protein Chem. 35, 213–321
33. Walsh, M., Stevens, F. C., Kuznicki, J., and Drabikowski, W. (1977) J. Biol. Chem. 252, 7440–7443
34. Johnson, B. A., Shirokawa, J. M., Hancock, W. S., Spellman, M. W., Basa, L. J., and Aswad, D. W. (1989) J. Biol. Chem. 264, 14262–14271
35. Ota, I. M., and Clarke, S. (1989) J. Biol. Chem. 264, 54–60
36. Murtaugh, T. J., Wright, L. S., and Siegel, F. L. (1986) J. Neurochem. 47, 164–172
37. Bischoff, R., and Kolbe, H. V. Y. (1994) J. Chromatogr. 662, 261–278
38. Liu, D. T. (1992) Trends Biotechnol. 10, 364–369
39. Gregori, L., Marriott, D., Putkey, J. A., Means, A. R., and Chau, V. (1987) J. Biol. Chem. 262, 2562–2567
40. Ziegenhagen, R., Goldberg, M., Rakutt, W.-D., and Jennissen, H. P. (1990) FEBS Lett. 271, 71–75
41. Babu, Y. S., Bugg, C. E., and Cook, W. J. (1988) J. Biol. Chem. 263, 191–204
42. Siegel, F. L. (1988) Adv. Exp. Med. Biol. 231, 341–351
43. Ziegenhagen, R., and Jennissen, H. P. (1988) Biochem. Hoppe-Seyler 369, 1321–1324
44. Borchardt, R. T., Eiden, L. E., Wu, B., and Rutledge, C. O. (1979) Biochem. Biophys. Res. Commun. 98, 199–207
45. Cicerelli, M. F., and Smith, L. D. (1987) Biochem. J. 245, 364–369
46. Kelly, G. M., Zelus, B. D., and Moon, R. T. (1991) J. Biol. Chem. 266, 5469–5473
47. Schrama, L. H., Lepperding, G., Meritz, A., Van den Engel, N. K., Marquart, A., Oestreich, A. B., Eggert, B. J. L., Hage, W. J., Richter, K., and Destree, O. H. J. (1997) Neurosci. 76, 635–652
48. Stadtman, E. R. (1990) Biochemistry 29, 6323–6331
49. Artigues, A., Farrant, H., and Schirch, V. (1993) J. Biol. Chem. 268, 13784–13790