The widely distributed protein-L-isoaspartyl, D-aspartyl carboxylmethyltransferase (EC 2.1.1.77) is hypothesized to play a role in the repair or metabolism of deamidated and isomerized proteins that are spontaneously generated during the aging of proteins in cells. The yeast two-hybrid system was used to identify proteins that potentially interact with the methyltransferase in a cellular processing pathway. Two cDNAs, both encoding calmodulin, were isolated from a human fetal brain cDNA library using the human methyltransferase as the bait. Enzymatic assays with purified components revealed a complex set of interactions between the methyltransferase and calmodulin. Calmodulin weakly stimulated protein carboxylmethyltransferase activity in vitro at concentrations of the two proteins reflecting their representation in mammalian brain. Calmodulin stimulation of methyltransferase was observed in both the presence and absence of calcium, although the effect was greater in the presence of calcium. Native calmodulin was not a substrate for the carboxylmethyltransferase, but deamidated variants of calmodulin act as substrates for the methyltransferase, with calculated \( K_m \) values of 3.6 and 8.6 \( \mu M \) for calcium-ligated and unliganded calmodulin, respectively. Both the effector and substrate interactions of calmodulin with the protein isoaspartyl methyltransferase likely contributed to the positive results obtained with the two-hybrid system.

Protein-L-isoaspartyl, D-aspartyl carboxyl methyltransferase (PCMT)\(^1\) (E.C. 2.1.1.77), a well-conserved enzyme detected in a broad spectrum of organisms from Escherichia coli to humans (1–6), specifically modifies L-isoaspartyl and D-aspartyl residues that arise in proteins due to spontaneous deamidation and racemization reactions involving asparagine and aspartate residues (7, 8). Because the formation of L-isoaspartate can adversely affect the stability and biological potency of many proteins (9–13), it has been proposed that methylation of the damaged protein initiates either the repair or degradation of the substrate. Experimental support for a structural repair function for PCMT has been obtained in purified systems using isoaspartyl peptides as substrates for the PCMT (14–16). The esterified isoaspartyl peptides formed by PCMT hydrolyze into both aspartyl peptides and isoaspartyl peptides, the latter of which can be remethylated by the PCMT. The nearly quantitative conversion of isoaspartyl to aspartyl forms of the peptides has been interpreted as evidence for a repair function, although the process lacks the efficiency characteristic of enzymatic processes, and no isoaspartyl residues are converted into asparaginyl residues, the likely origin of most isoaspartyl sites in cellular proteins (2).

Because of the structural complexity of protein substrates for the PCMT, very little information is available regarding the fate of isoaspartyl residues in protein substrates. Deamidation of some proteins, including calmodulin (11) and the bacterial HPr phosphocarrier protein (17), generates isoaspartyl forms that act as substrates for the PCMT. Methylation of these isomerized calmodulin and HPr variants results in the partial restoration of enzymatic activity, suggesting that PCMT may play a role in functional repair of damaged proteins. In the case of the HPr protein, the increase in enzymatic activity following methylation has been shown to be accompanied by the conversion of the affected isoaspartyl residues to aspartyl residues but not to the original asparaginyl residues (17). These results demonstrate that PCMT-catalyzed methylation is insufficient in itself to effect full structural or functional repair of substrate molecules, raising the possibility that additional cellular components could interact with PCMT in a protein repair pathway in vivo.

Due to the chemical instability of protein methyl esters generated by PCMT, it has not been possible to follow the fate of modified proteins subsequent to their methylation in vivo or to identify additional cellular activities that participate in potential processing/repair pathways. Because of these limitations, we sought an alternative approach to further elucidate the role of PCMT in the cellular processing of altered proteins. In an effort to identify proteins that interact with PCMT, we have used the yeast two-hybrid system to screen a human fetal brain cDNA library. A brain library was chosen for these studies because of the high concentration of the PCMT in brain (18) and because of the severe neurological defects observed in mice lacking the PCMT (19). The yeast two-hybrid system is a highly sensitive method for detecting protein-protein interactions that has been successfully used to detect weak interactions undetectable by other physical methods such as immunoprecipitation (20, 21). We describe here the isolation of two cDNAs, both encoding full-length CaM, that specifically interact with human PCMT. The significance of the interaction has been confirmed by enzymatic measurements of the CaM dependence of carboxyl methylation reactions. The stimulation of PCMT activity by CaM is distinct from the substrate activity of deamidated CaM, highlighting the complexity of PCMT-CaM interactions. The value of the yeast two-hybrid system for identifying weak interactions and the physiological significance of the PCMT-CaM interactions are discussed.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Molecular manipulations were conducted according to standard protocols (22, 23). A full-length cDNA clone (24)
encoding the acidic isozyme of human PCMT (hPCMT) was kindly provided by Dr. Steven Clarke (Dept. of Chemistry and Biochemistry, University of California at Los Angeles, CA). The parent shuttle vectors for the yeast two-hybrid analysis, the human fetal brain cDNA library, and the nonspecific bait constructs were kindly provided by the laboratory of Dr. Roger Brent (Massachusetts General Hospital, Boston, MA) (25, 26). The bait construct consists of a polymerase chain reaction-generated EcoRI/SalI fragment of hPCMT cDNA ligated into the EcoRI/SalI sites of the 2 μm HIS 3 plasmid pEG202 (26). The resulting fusion protein consists of the DNA binding domain of LexA fused to hPCMT under the control of the constitutive promoter ADH1. The construct was verified by DNA sequencing using standard procedures (29). The library was constructed using the 2 μm TRP1 pJG4–5 plasmid (27), in which cDNA sequences are fused to the B42 “acid patch” activation domain (28), and expression of the fusion proteins is controlled by the inducible GAL1 promoter.

**Strains—**Saccharomyces cerevisiae strain EGY48 (MATa, ura3, trpl, his3, lexAop-LEU2) was used for all two-hybrid analyses (27, 29). EGY48 contains six LexA binding sites replacing the upstream activating sequence of the LEU2 gene. EGY48 was sequentially transformed with the pSH18–34 lacZ reporter and pEG202-hPCMT bait plasmids to generate a LexA selection strain for library screening. Therefore, growth on leucine-deficient media serves as an indication of an interaction between the LexA-PCMT bait protein and a B42 fusion prey protein, and this interaction can be confirmed using the lacZ reporter gene. E. coli SURE-2 cells (Stratagene, La Jolla, CA) were used for plasmid manipulation in bacteria.

**Transformation and Library Screening—**Yeast interaction trap selections (Fig. 1) were performed essentially as described (27, 29). Yeast transformants were generated using a modified lithium acetate transformation protocol (30) and grown at 30 °C using the appropriate synthetic media (29). Yeast EGY48 cells already harboring the pEG202-hPCMT plasmid and a pSH18–34 lacZ reporter plasmid were transformed with a human fetal brain cDNA library. The transformation mix was plated on synthetic complete medium containing glucose (2%) and lacking uracil, histidine, and tryptophan. All transformants were pooled and stored at −80 °C. An aliquot was grown in synthetic liquid medium containing galactose (2%) and raffinose (1%) and lacking uracil, histidine, and tryptophan for 48 h to induce expression of the library cDNAs. The transformation efficiency was 105 transformants/μg of library DNA. Approximately 2 × 108 transformants were plated onto synthetic complete medium that included galactose (2%) and raffinose (1%) and lacking uracil, histidine, tryptophan, and leucine to select for colonies that express cDNA-encoded proteins that interact with the PCMT bait protein. After 3 days, colonies were replica-plated to fresh plates of the same medium. After an additional 2 days, colonies were transferred to synthetic medium containing glucose (2%) and lacking uracil, histidine, and tryptophan to switch off synthesis of activation-tagged cDNA-encoded protein and allow residual galactose-induced mRNA and protein to decline to undetectable (basal) levels. After 2 days, colonies were replica-plated onto four new selection plates to test for galactose-dependent lacZ expression and Leu protrophy. Primary positives were replicated to fresh plates of the same medium. Secondary positives were identified and collected for further analysis. The cDNA plasmids were rescued and reintroduced into the original selection strain and into four related strains expressing nonspecific LexA selection strain as well as several negative control strains described under “Experimental Procedures.” Galactose-dependent LexA- and lacZ+ candidates were selected for further analysis.

**Methyltransferase Assay—**PCMT assays were carried out at 37 °C in 25 mM MES, pH 6.5. The more acidic isozyme of PCMT was purified from calf brain as described by Awad and Deight (34) and used in all experiments. Assay mixtures (25 μl) contained final concentrations of 0.44 mM ovalbumin, 22 mM PCMT, 2.5 μM S-adenosyl-L-[methyl-3H]methionine (NEN Life Science Products) adjusted to a final specific activity of 1 Ci/mmol with nonradioactive S-adenosyl-L-methionine (Sigma). Protein carboxyl ([3H]methionyl esters were quantified as trichloroacetic acid-precipitable material converted to [3H]methanol by base treatment, as described previously (36). In experiments in which calcium or EGTA was included, PCMT was preincubated with calcium or EGTA for 30 min on ice. Each assay was done in triplicate and included a negative control lacking substrate, representing the assay background.

**RESULTS**

**Two-hybrid Screening—**The yeast two-hybrid system was used to screen for proteins in human brain that could bind to hPCMT (Fig. 1). In these experiments, a physical association between hPCMT fused to LexA and a second protein fused to the B42 transcription activation domain was detected by the transcription of two reporter genes, LEU2 and lacZ, that contain upstream LexA binding sites (27, 29). Approximately 2 × 107 transformants were screened, and of these, 36 clones showed galactose-dependent LexA protrophy and lacZ expression on X-gal indicator plates following secondary screening. To determine whether the cDNA-encoded proteins specifically interacted with the LexA-hPCMT library plasmids were rescued from these 36 clones (30) and used to transform the original selection strain as well as several negative control strains expressing Max-, Drosophila Bicoid-, Cdk 1-, and Cdi 1-LexA fusion proteins as bait. Three clones encoding proteins that
Protein Carboxylmethyltransferase-Calmodulin Interactions

Calmodulin Stimulation of PCMT Activity in Vitro—Calmodulin, a ubiquitous and abundant protein in eucaryotic cells, is involved in the regulation of many cellular processes controlled by calcium-dependent signaling pathways (36). Calmodulin has been implicated in the calcium-dependent activation of a large number of enzymes (for a review, see Ref. 37), including myosin light chain kinase (38), the plasma membrane Ca2+-ATPase (39), the CaM-dependent kinases (40), and nitrile oxide synthetase (41). In the absence of calcium, Calmodulin also binds to a number of cellular proteins, including the abundant neuroprotein neuromodulin (42), which is associated with neuronal growth and neurite extension, and the 110-kDa myosin from intestinal microvilli (43, 44). There are no previous reports of PCMT activation by Calmodulin, although the distribution of the two proteins in mammalian tissues is similar (18, 36) and Calmodulin serves as a physiological substrate for PCMT (45–49). Calmodulin stimulation of PCMT-catalyzed methylation of ovalbumin in a concentration-dependent manner when both proteins are present at physiological concentrations but that significant molar excesses of Calmodulin are required for maximal stimulation of PCMT activity.

Carboxyl Methylation of Deamidated CaM—CaM deamidation to a methyl-accepting substrate for PCMT occurs with a half-time of 8–9 days at pH 7.4 and 37 °C (11, 51) in vitro. Therefore, it is expected that most of the CaM expressed in yeast cells is in the native form, but the sensitivity of the two-hybrid system does not seem to preclude detection of PCMT interactions with a minor subpopulation of CaM molecules that could be present in yeast. To evaluate this possibility, kinetic parameters were determined for the methylation of deamidated CaM catalyzed by the PCMT. Deamidated rCaM was prepared as described under “Experimental Procedures” and analyzed by nondenaturing polyacrylamide gel electrophoresis (Fig. 3). Densitometric analysis of the gel indicated that the deamidated CaM variants represented approximately 78% of total protein, similar to results obtained in previous studies (11, 45). Fig. 4 shows the kinetic data obtained using deamidated rCaM as the substrate for the PCMT in both the presence and absence of calcium. In the presence of 3 mM EGTA, CaM stimulated PCMT by approximately 19%. A larger stimulation of approximately 42% was observed in the presence of 3 mM calcium. In control experiments without CaM, PCMT activity was unaffected by both the presence and the absence of calcium (data not shown). Together, the results demonstrate a small but reproducible stimulation of PCMT activity by CaM, which is further enhanced in the presence of calcium.

Table I

| CaM concentration (mM) | Initial velocity (pmol methyl groups transferred/min·mg of protein) | Stimulation (%) |
|------------------------|---------------------------------------------------------------|----------------|
| 0                      | 12,300 ± 310                                                  | 0              |
| 0.1                    | 14,700 ± 150                                                 | 30.1           |
| 0.5                    | 16,500 ± 220                                                 | 42.0           |
| 1.0                    | 16,200 ± 800                                                 | 39.5           |
| 10                     | 17,400 ± 2500                                                | 49.9           |
| 20                     | 17,600 ± 800                                                 | 51.8           |

* The initial velocity of ovalbumin methylation catalyzed by bovine brain PCMT was measured in the presence of the indicated concentrations of CaM. Rates of methylation were calculated by linear regression analysis of the incorporation data. A background value obtained in parallel without substrate was subtracted from each measurement.

Because deamidated CaM would be expected to represent only a small subpopulation of CaM molecules overexpressed in yeast cells, we determined whether native CaM could also interact with PCMT by measuring the effect of native rCaM on PCMT-catalyzed methylation of ovalbumin (Fig. 2). The concentration of native rCaM used in these experiments was 0.5 μM, a value near its physiological concentration (36) and also within the range of expression for library proteins in the yeast two-hybrid system (25). Under the conditions in our assay, rCaM shows no methyl-accepting activity in either the presence or absence of calcium (data not shown; see also Ref. 50). As shown in Fig. 2, CaM stimulated ovalbumin methylation catalyzed by the PCMT in both the absence and presence of calcium. In the absence of EGTA, CaM stimulated PCMT by approximately 19%. A larger stimulation of approximately 42% was observed in the presence of 3 mM calcium. In control experiments without CaM, PCMT activity was unaffected by both the presence and the absence of calcium (data not shown). Together, the results demonstrate a small but reproducible stimulation of PCMT activity by CaM, which is further enhanced in the presence of calcium.

Fig. 2. Effect of native rCaM on PCMT catalytic activity. Ovalbumin methylation was measured as described under “Experimental Procedures” in the absence of CaM (○), in the presence of 0.5 μM rCaM and 3 mM EGTA (+rCaM/EGTA (■)), or in the presence of 0.5 μM rCaM and 3 mM calcium (+rCaM/EGTA/Calcium (□) at 37 °C for the times indicated. Each value represents the mean ± S.E. of three measurements. A background value obtained in parallel without substrate was subtracted from each measurement.

Stimulation = 100 × (1 - V0/Vc)
Initial rates of methylation using various concentrations of deamidated CaM as substrate were measured in the presence of 3 mM EGTA as described under “Experimental Procedures.” Samples containing 3 μg of protein were analyzed by nondenaturing polyacrylamide gel electrophoresis as described previously (11, 33). Gels were stained with Coomassie Blue. dCaM, deamidated CaM variants.

![Image](63x352 to 283x553)

**Fig. 3.** Nondenaturing gel electrophoresis of CaM variants generated by prolonged incubation in vitro. Recombinant chicken CaM was incubated at 37 °C for 14 days in the presence of either 1 mM CaCl₂ or EDTA as described under “Experimental Procedures.” Samples containing 3 μg of protein were analyzed by nondenaturing polyacrylamide gel electrophoresis as described previously (11, 33). Gels were stained with Coomassie Blue. dCaM, deamidated CaM variants.

**FIG. 4.** Carboxyl methylation of deamidated CaM catalyzed by the PCMT. Initial rates of methylation using various concentrations of deamidated CaM as substrate were measured in the presence of 3 mM calcium (○) and in the presence of 3 mM EGTA (●) as described under “Experimental Procedures.” Reactions were carried out for 15 min, during which the rate of incorporation was constant. Each value represents the mean of triplicate determinations corrected for a background value obtained without substrate. The incorporation curve represents the results of a nonlinear regression of the data using the Michaelis-Menten equation. Calculated values using calcium-liganded substrate indicate a \( K_m \) value of 8.6 μM and a \( V_{max} \) value of 10,000 methyl groups transferred/min-mg of protein. Values calculated for unliganded CaM include a \( K_m \) value of 3.6 μM and a \( V_{max} \) value of 15,000 methyl groups transferred/min-mg of protein.

unliganded CaM serve as substrates for the enzyme, although the unliganded CaM serves as the better substrate of the two forms. Kinetic analysis of the incorporation data using standard Michaelis-Menten assumptions results in calculated \( K_m \) values of 3.6 and 8.6 μM for unliganded CaM and calcium-liganded CaM, respectively. The maximum velocity of the PCMT with the two forms is also significantly greater with unliganded deamidated CaM as substrate. The calculated \( V_{max} \) values are 15,000 and 10,100 pmol methyl esters formed/min-mg of enzyme for the unliganded and calcium-liganded forms of the deamidated CaM, respectively. The specificity constant (\( V_{max}/K_m \)) calculated from these data is 3.5 higher for the unliganded form than the calcium-liganded form, suggesting that unliganded CaM is the preferred substrate for the enzyme.

**DISCUSSION**

In this study, we used the yeast two-hybrid system to identify proteins from brain that interact with the PCMT in vitro. This approach, which is capable of detecting very weak interactions, has identified CaM as a specific interactor. The significance of the PCMT-CaM interaction was evaluated by measuring CaM activity as both an activator and as a substrate for PCMT in vitro. These results indicate that the interaction detected in the yeast two-hybrid system is complex and may reflect more than a single kind of interaction between these proteins.

Measurements of PCMT activity in the presence and absence of CaM (Fig. 2) indicate that CaM weakly stimulates PCMT catalytic activity. Stimulation was observed in both the absence and presence of calcium, although the effect was more pronounced with calcium. The magnitude of the stimulation produced by CaM, however, was much less dramatic than those commonly observed for CaM-dependent activations and is unusual in its very slight calcium selectivity. CaM interacts with most effector proteins in a strongly calcium-dependent manner (36, 38, 41) and with a smaller number in a calcium-independent manner. Calcium-independent interactions have been described for the abundant neuroprotein neuromodulin (42) and the 110-kDa myosin from intestinal microvilli (43, 44). In general, the calcium-independent interactions of CaM with proteins are characterized by lower affinity interactions, with \( K_d \) values on the micromolar level, compared with the nanomolar affinities characteristic of calcium-dependent interactions (52). Furthermore, some calcium-independent interactions can be weakly affected by the presence of calcium. Neuromodulin, for example, binds CaM with an affinity of 0.23 μM in the absence of calcium and with an affinity of 1 μM in the presence of calcium (42). This affinity of neuromodulin (also known as GAP-43) for CaM is sufficiently strong to allow detection in the yeast two-hybrid system, as recently reported (53). From the data presented in Fig. 2 and Table I, it appears that the interaction of PCMT with CaM is similar to the previously described calcium-independent interactions, although the PCMT sequence does not contain a putative consensus binding site for Ca-independent interactions of CaM (52).

Although the effect of CaM on PCMT catalytic activity is not large, it is most likely physiologically significant. Both CaM and PCMT are widely distributed in eucaryotes and are present in all tissue types of vertebrates (18, 36). Particularly high concentrations of both proteins are found in brain, including neuron-enriched regions of the hippocampus and cerebral cortex. The assays used here have been adjusted to reflect the concentrations and molar ratios of the two proteins in brain tissue. The results from these assays suggest that CaM weakly activates PCMT in vitro and that this stimulation is marginally affected by alterations in cellular calcium. This small but reproducible stimulation observed in vitro may underestimate the true significance of the interaction, however, if CaM and PCMT interact as components of larger protein complexes in vivo. These proteins could, for example, be involved in a processing pathway downstream of PCMT for the removal or repair of damaged proteins in the cell.

The effector interaction of CaM with PCMT is clearly distinct from the substrate interaction of deamidated CaM with PCMT. Previous experiments have shown that prolonged incubation of purified CaM in the absence of calcium at physiological temperature and pH is accompanied by large increases in its methyl-accepting activity (11). This methyl-accepting activity has been mapped to multiple isospartyl sites in the calcium-binding loops and in flexible regions near the N terminus and at positions 78 and/or 80 (33, 49, 54, 55). Similar sites are methylated in biosynthetically labeled CaM purified from intact erythrocytes (49), suggesting that novel variants produced in vitro reflect naturally occurring variants of CaM. Because the
concentration of calcium in cell cytosol is typically in the submicromolar range, most cellular CaM would be expected to be present in the unliganded form that more readily deamidates than calcium-ligated CaM. In vivo, these deamidated forms represent only a small fraction of the total CaM in intact cells, as evidenced by the extremely low stoichiometries of CaM methylation observed in isotopic tracer studies. In Xenopus oocytes, for example, carboxyl methylated CaM represents 0.02% of the total oocyte CaM (45). Similarly, carboxyl methylated CaM is estimated to represent 0.03% of human erythrocyte CaM (46). The large discrepancy between the proportion of methyl-accepting CaM in vivo and in vitro is consistent with the active metabolism of deamidated CaM in living cells. Likewise, we would expect that deamidated CaM variants would represent only a minor fraction of the CaM-fusion proteins overexpressed in yeast cells used for the two-hybrid system. It should be noted, however, that yeast is one of the few cell types in which it has proven difficult to detect PCMT activity (6), and sequences homologous to conserved PCMT regions have not been identified in searches of the yeast genome data base.2 It is not clear whether yeast cells possess an alternative pathway for processing isoaspartyl residues that appear in proteins, which would prevent the accumulation of isoaspartyl-containing proteins.

To determine whether the small subpopulation of yeast CaM expected to exist in the deamidated form might contribute to the interaction(s) detected in the two-hybrid system, we have determined the kinetic parameters describing both calcium-ligated and unliganded deamidated CaM as substrates for the PCMT. As shown in Fig. 4, both forms of deamidated CaM can serve as substrates, but the unliganded form has a significantly higher affinity for the PCMT. A similar effect has been observed for a deamidated form of calbindin D24, another intracellular calcium-binding protein that contains an isoaspartyl residue in one of its two calcium binding sites (56). In the absence of calcium, this protein exhibits a 10-fold increase in affinity for PCMT. These increases in methyl-accepting activity observed with CaM and calbindin upon calcium removal most likely reflect conformational changes in the protein that expose isoaspartyl residues to the PCMT, including isoaspartyl residues that complex to calcium in the calcium binding sites (33).

From the data presented here, it appears that CaM may well interact with the PCMT as both an activator and as a substrate in the yeast two-hybrid system, depending on the distribution of the overexpressed CaM between native and deamidated forms. In the two-hybrid system, most fusion proteins are typically expressed at levels within the range of 50 nM to 1 M, and the cDNA-encoded proteins are present at nuclear concentrations of approximately 1 M (27, 29). Given the likelihood that deamidated variants represent only a small fraction of CaM-fusion proteins produced in yeast cells and the higher affinity of PCMT for native (Table I) than for deamidated (Fig. 4) CaM, it is probable that the stimulatory interactions of native CaM with PCMT are principally responsible for our positive results with the two-hybrid system. At the high expression levels achieved in yeast cells, however, substrate interactions of deamidated CaM and PCMT could also contribute to the results, confirming CaM as a significant physiological methyl-acceptor (45–48) and validating the use of CaM as a model substrate for PCMT (45).

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2 S. Clarke, personal communication.