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Regulation of Synthesis and Degradation of Rat Adrenal Phenylethanolamine *N*-methyltransferase

IV. Synergistic Stabilization of the Enzyme against Thermal and Tryptic Degradation by S-Adenosylmethionine and Biogenic Amine Substrates

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SUMMARY

MASOVER, S. J., D. M. BERENBEIM, AND R. D. CIARANELLO. Regulation of synthesis and degradation of rat adrenal phenylethanolamine *N*-methyltransferase. IV. Synergistic stabilization of the enzyme against thermal and tryptic degradation by S-adenosylmethionine and biogenic amine substrates. *Mol. Pharmacol.* **16**: 491-503 (1979).

The effect of several biogenic amines that are substrates for adrenal phenylethanolamine N-methyltransferase (PNMT) on thermal and tryptic stability of the enzyme is described. While all substrates tested were somewhat effective in stabilizing the enzyme against tryptic degradation, only phenylethanolamine, phenylethylamine and octopamine were effective in stabilizing PNMT against thermal denaturation. Stabilization of PNMT was maximal when the *m* and *p* positions of the phenylethylamine nucleus were unsubstituted. Thus phenylethylamine was the most effective stabilizing compound studied, while norepinephrine was least effective. Epinephrine was completely ineffective in stabilizing the enzyme against proteolytic or thermal breakdown. The interaction of S-adenosylmethionine and substrate in protecting PNMT against thermal and tryptic degradation was also tested. While both substrate and S-adenosylmethionine protect the enzyme against tryptic proteolysis when present singly, the combination of the two compounds affords a degree of stabilization that is substantially greater than that seen by either compound alone. Moreover, the presence of substrate increases the affinity of the enzyme for S-adenosylmethionine, thus reducing the concentration of S-adenosylmethionine required for maximal stabilization of the enzyme. S-adenosylmethionine does not increase the affinity of PNMT for norepinephrine, nor does it augment the stabilization afforded by substrate. These results suggest that *in vivo* PNMT proteolysis is controlled by the constituents of the PNMT reaction, particularly S-adenosylmethionine. We postulate that interaction of the transferase with its substrate and with S-adenosylmethionine alters the spatial conformation of the protein in such a way that proteolytically vulnerable sites on the molecule are protected.

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INTRODUCTION

Previous reports in this series (1-3) have shown that glucocorticoids control the

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steady-state levels of rat adrenal PNMT[2] by regulating the rate of *in vivo* proteolysis of the enzyme. Further investigation has disclosed the existence of an endogenous compound which controls the thermal and tryptic degradation of PNMT, and whose levels are decreased or abolished by hypophysectomy and restored by glucocorticoids. Isolation and characterization of this compound show it to have properties similar to those of S-adenosylmethionine. S-adenosylmethionine stabilizes PNMT *in vitro* against both thermal and tryptic degradation, while administration of this compound *in vivo* to hypophysectomized rats results in partial restoration of PNMT levels.

These results led us to speculate that the proteolysis of PNMT might be regulated *in vivo* by the levels of SAM, which were in turn regulated by adrenal glucocorticoids. However, the problem is considerably more complicated than this simple speculation. S-adenosylhomocysteine, the demethylated analogue of SAM, also stabilizes PNMT to tryptic proteolysis, although not to thermal denaturation (3). Moreover other compounds whose levels are altered by hypophysectomy may also stabilize PNMT. Any model, then, which attempts to explain the regulation of PNMT proteolysis *in vivo* must take into account the effects of hypophysectomy on SAM, on SAH, and on other compounds which might stabilize the enzyme.

Among this last group of compounds are the biogenic amine substrates of PNMT, and the reaction product, epinephrine. During the course of our studies on S-adenosylmethionine it became apparent that, if one reaction constituent (SAM), or its product (SAH), could influence PNMT stability, then other compounds which were reaction constituents might function in a stabilizing role as well. PNMT catalyzes the formation of epinephrine, a bimolecular reaction in which SAM and norepinephrine participate. Each reaction constituent has a discrete binding site or sites, but epinephrine, the reaction product, may have a binding site as well because it is a noncompetitive inhibitor of the PNMT reaction (4). Thus the possibility existed that one, two or even three reaction constituents might play a role in regulating PNMT degradation *in vitro* and, by extension, enzyme proteolysis *in vivo*.

[2] The abbreviations used are: PNMT, phenylethanolamine N-methyltransferase SAM, S-adenosylmethionine; SAH, reaction product of SAM, S-adenosylhomocysteine.

MATERIALS AND METHODS

Enzyme assays. Phenylethanolamine N-methyltransferase activity was measured in supernatant preparations from normal rats, and in partially purified preparations obtained from bovine adrenal medullae. When the rat adrenal enzyme was used, the animals were killed by cervical dislocation and the adrenals were removed. The glands were homogenized in 5 ml per pair 50 mm Tris-HCl buffer, pH 7.4. Following centrifugation at 37,000 × *g*, the supernatants were dialyzed overnight in the cold against several changes of homogenizing buffer to remove endogenous stabilizing factors, as previously described (2). PNMT activity was assayed using 100 μl portions of the supernatant incubated with 2 nmol [^{14}C-*methyl*]- or [^{3}-H-*methyl*]-S-adenosylmethionine, and appropriate amounts of nonradioactive biogenic amine substrate.

Following incubation at 37° for 30–60 min, the radioactive N-methylated product was extracted into 5 ml of a suitable organic solvent system, from which 3 ml portions were taken for liquid scintillation counting. To minimize the effects of competitive inhibition, the same biogenic amine substrate being tested for stabilization efficacy was used to measure the residual PNMT activity remaining after denaturation (see below). The substrates and the organic solvent systems used to extract methylated product were: phenylethylamine and phenylethanolamine : toluene-isoamyl alcohol, 97:3; octopamine and noremetanephrine : toluene-isoamyl alcohol, 3:2; norepinephrine : NaCl-saturated butanol.

In later experiments PNMT from bovine adrenal medulla was used as enzyme source. The enzyme was purified through the Sephadex G-100 step, as previously described (5). Ten microliter portions of the Sephadex-purified material was diluted

with 90 μl of 50 mM Tris-HCl buffer, pH 7.4, before being added to the assay system.

Thermal and Tryptic Denaturation Studies. Studies on thermal and tryptic stability of PNMT were performed on dialyzed rat adrenal supernatants, or on the partially purified bovine enzyme, as previously described (2, 3). The effects of varying concentrations of biogenic amine substrate were tested by adding the compound to the enzyme mixture prior to heating or to tryptic proteolysis. The concentration ranges chosen were determined by estimating the K_m of each substrate for PNMT and selecting an appropriate range of concentrations on either side of the K_m. At the termination of heating or of tryptic digestion, residual PNMT activity was determined by adding 2 nmol radioactive SAM and an appropriate amount of the biogenic amine substrate so that all tubes were assayed at a constant substrate concentration. The final concentrations of amine substrates were: phenylethanolamine 1400 μM, octopamine 9 μM, normetanephrine 600 μM, norepinephrine 35 μM. When phenylethylamine and epinephrine were being tested, phenylethanolamine at a final concentration of 1400 μM was used as the PNMT substrate.

Isotopes. [^{14}C-*methyl*]S-adenosylmethionine, 60 mCi/mMol, or [^{3}H-*methyl*]S-adenosylmethionine was purchased from Amersham-Searle (Des Plaines, Ill.). When tritiated S-adenosylmethionine was used, the specific activity was adjusted to 60 mCi/mMol by the addition of unlabeled S-adenosylmethionine (Sigma Chemical Co., St. Louis), which had first been purified by ion-exchange chromatography (6).

Statistical analysis. The determination of the rate constants of denaturation in the thermal and tryptic systems, as well as the statistical tests used to determine differences between the regression coefficients, have been described previously (2).

RESULTS

Comparison of thermal and tryptic stabilities for rat and bovine PNMT. The earlier studies in this series (1-3) were performed on crude adrenal supernatants from hypophysectomized rats. Subsequent studies revealed that dialysis of normal rat adrenal preparations removed the endogenous stabilizing factors, yielding a PNMT preparation with much higher enzyme activity but with thermal and tryptic susceptibility identical to that of enzyme from hypophysectomized rats. Since bovine adrenals are an even richer source of the enzyme, we investigated the thermal and tryptic characteristics of PNMT purified from this source. The behavior of bovine PNMT in the thermal and tryptic denaturation systems was compared to that of PNMT from dialyzed preparations of normal rats and to crude preparations from hypophysectomized rats to ensure that no artifacts were being introduced by the change in enzyme sources. Purified bovine PNMT behaved identically to rat PNMT with regard to its K_m and EC_{50} for SAM, its thermal half-life and its stabilization by S-adenosylmethionine (Table 1). Accordingly, PNMT from bovine adrenals was deemed an appropriate source of enzyme material for use in these studies.

TABLE 1

Comparison of properties of rat and bovine adrenal PNMT

Adrenal supernatants from hypophysectomized or normal rats were prepared as described in METHODS. PNMT from bovine adrenal medulla was purified as previously described (5); the purification protocol calls for dialysis twice during the procedure. For the enzyme from each preparation, the K_m for phenylethanolamine and for S-adenosylmethionine were determined by the method of Lineweaver and Burk. The EC_{50} of SAM was determined by adding varying concentrations of SAM to PNMT before heating to 50°, as described in the previous report (3). The EC_{50} was defined as that molar concentration of SAM required to produce a half-maximal stabilization of the enzyme against thermal denaturation. The half-life at 50° was determined by heating the enzyme in the absence of any stabilizing agents and determining loss of enzyme activity over time, as described in (2, 3).

Enzyme source	K_m SAM	K_m phenylethanolamine	EC_{50} SAM	$t_{1/2}$, 50°
	(μM)	*(μM)*	*(μM)*	*(min)*
Hypophysectomized rat	2.66	71.4	2.80	2.83 ± 0.15
Rat, normal (dialyzed)	2.75	76.9	2.95	2.54 ± 0.23
Bovine	2.86	67.0	2.71	2.55 ± 0.14

Michaelis-Menten determinations. Four biogenic amines known to be PNMT substrates (phenylethanolamine, norepinephrine, normetanephrine, octopamine) were used to test whether these compounds stabilized PNMT against *in vitro* denaturation. Phenylethylamine, a compound with very limited substrate activity, was also tested. To determine the concentration range to be tested for each compound, the Michaelis-Menten contant was first determined. Our findings agreed with those already in the literature, except that in our hands octopamine has a slightly lower K_m than norepinephrine (usually norepinephrine has been reported to have a higher affinity for the bovine enzyme than octopamine [4]). Phenylethylamine, which is ordinarily not considered a PNMT substrate, was found to have very limited, but measurable substrate activity.

Effects of PNMT substrates on enzyme thermal stability. Each of the above compounds was examined for its efficacy as a stabilizer of PNMT against thermal denaturation (Table 2). The stabilization properties of S-adenosylmethionine have already been described (3), but are included in Table 2 for reference purposes.

S-adenosylmethionine was the most effective stabilizer against thermal denaturation of the compounds tested. The maximal stabilization seen with S-adenosylmethionine was 2.09-fold. The rank order of stabilization with the amine substrates was phenylethanolamine > octopamine > normetanephrine > norepinephrine. It is doubtful that the degree of stabilization seen with normetanephrine is statistically significant. Norepinephrine, the natural PNMT substrate, showed no thermal stabilization. Epinephrine, the product of the PNMT reaction *in vivo*, was also ineffective as a stabilizer against thermal denaturation.

Effect of PNMT substrates on enzyme tryptic stability. We have observed throughout these studies a high degree of congruence between results obtained in the thermal denaturation system and data from the tryptic degradation system. However, a disparity was observed with S-adenosylhomocysteine (3), which was ineffective in protecting PNMT against thermal denaturation but highly effective in inhibiting the tryptic proteolysis of the enzyme. This suggested that the chemical properties of a putative stabilizer at 50° might introduce falsely negative artifacts into our conclusions, and that a given compound should be tested in both the tryptic and thermal systems before a judgment was made about its stabilization efficacy.

Accordingly, the same group of biogenic amine substrates was tested for stabilization of PNMT against tryptic proteolysis. Once again SAM was included for reference

TABLE 2

Effect of various substrates on PNMT thermal stability

The stabilization constant and maximum stabilization of each of the above compounds was tested. Each compound was incubated over a wide concentration range with bovine PNMT. Thermal denaturation at 50° was carried out as previously described. K_s, the stabilization constant, was determined from a plot of $1/k$ versus $1/S$, where k is the first-order rate constant of thermal denaturation and S is the substrate concentration (see figure 1 and [3]). The method is analogous to the K_m determination of Lineweaver and Burk. The maximum stabilization is defined as the maximum half-life seen in the presence of the stabilizer divided by the half-life observed in its absence. Thus a 1.00 value means a compound gave no significant degree of stabilization at any concentration. Numbers in parentheses are the times an experiment was performed.

Substrate	K_s	Maximum stabilization (fold)
	(μM)	*(mean ± SEM)*
S-adenosylmethionine (10)	0.481	2.09 ± 0.06
Phenylethylamine (2)	——	1.00 ± 0.08
Phenylethanolamine (4)	121.000	1.60 ± 0.02
Octopamine (4)	0.51	1.42 ± 0.03
Normetanephrine (2)	50.000[a]	1.19 ± 0.02
Norepinephrine (2)	——	1.00 ± 0.08
Epinephrine (8)	——	1.00 ± 0.06
None	——	1.00 ± 0.12

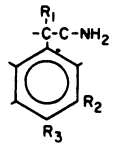
[a] The degree of stabilization seen with normetanephrine was barely significant. The accuracy of the K_s value obtained, therefore, is suspect. As an additional control, a mock experiment was carried out in which five separate thermal denaturation curves for PNMT were obtained in the absence of any substrate ("None"). The variation (SEM) in the mean half-life obtained was ± 12%, thus making it unlikely that normetanephrine is exerting any meaningful stabilization of the enzyme.

purposes, as was SAH. The data from the tryptic studies were similar to those seen in the thermal degradation experiments, with some important exceptions (Fig. 1). All compounds tested except epinephrine were effective stabilizers against tryptic proteolysis. For those compounds that stabilized the enzyme against both tryptic and thermal degradation, a striking congruence of K_s values between both systems was seen, and the rank order of stabilizing efficacy was almost identical between the two. These findings suggested that the chemical properties of a putative stabilizer at 50° had to be considered in evaluating the outcome of the thermal degradation experiments. (For example, norepinephrine and SAH are readily oxidized at 50°, while phenylethylamine is highly volatile.) Thus it is important in these types of studies to carry out both thermal and tryptic studies, rather than thermal studies alone. We have observed several cases where compounds that were ineffectual thermal stabilizing agents were effective against tryptic proteolysis, but we have never observed the opposite case: compounds effective as stabilizers against heat have always proved effective as stabilizers against trypsin.

Structure-activity relationship in tryptic stabilization among the biogenic amines. The results of the thermal and tryptic stabilization studies suggested a relationship between the structure of the biogenic amine substrate and the stabilization of PNMT against denaturation. These results are shown in Figure 1. They suggest two major points: first, that affinity of the biogenic amine for PNMT as measured both by K_s and by K_m depends on the presence of a hydroxyl group in the R_1 (β) position. It has been known for many years that the ability of a substituted phenylethylamine derivative to act as a PNMT substrate depends on its having a β-OH moiety (7). Our results confirm this and also show that the affinity of a stabilizing compound for the enzyme similarly depends on its having a hydroxyl group in the β- position.

Second, stabilization efficacy of a given biogenic amine is maximal when the *m* and *p* positions on the ring are unsubstituted. Thus phenylethylamine and phenylethanolamine, both of which have lower affinity for PNMT than the other substrates, are the most effective stabilizers in the series tested. Addition of an —OH group at the *p* position (octopamine) dramatically lowers the efficacy of the compound as a stabilizer, while additional substitution in the *m* position further reduces it. There appears to be a complete dissociation between affinity of a compound for the enzyme, which depends on β-substitution, and stabilization efficacy, which depends on the degree of substitution of the ring. The ring substituents are not as important in determining V_{max} as they appear to be in stabilization, however.

Thus the substrate activity of a biogenic amine is maximal when hydroxyl groups are present in the β, *m* and *p* positions



Compound	R_1	R_2	R_3	K_m (μM)	V_{max}	K_s (μM)	Maximum stabilization (fold)
Phenylethylamine	H	H	H	479	0.02	174	4.59
Phenylethanolamine	OH	H	H	67	0.25	66	4.69
Octopamine	OH	H	OH	2.5	0.13	0.5	2.33
Norepinephrine	OH	OH	OH	4.9	0.77	2.0	1.91
Normetanephrine	OH	OCH_3	OH	118	0.18	52	1.90

FIG. 1. *Structure activity relationship among several PNMT substrates*

(norepinephrine), but stabilization activity is maximal when all three positions are unsubstituted (phenylethylamine), or when only the β-position is substituted (phenylethanolamine). Because norepinephrine is the naturally-occurring substrate for PNMT, these findings call into question the importance of norepinephrine as the major *in vivo* regulator of PNMT proteolysis (particularly because its levels are unaffected by hypophysectomy) (8) and suggest that relatively more importance should be ascribed to S-adenosylmethionine.

Interaction between SAM and substrate in PNMT stabilization. Both S-adenosylmethionine and some of the PNMT substrates are highly effective stabilizers of PNMT against both tryptic and thermal denaturation. Because S-adenosylmethionine and substrate bind to different sites on the enzyme (9), we investigated whether or not there was any interaction between these sites. Such interaction might provide greater protection of the enzyme when both constituents were bound than that seen with binding of either molecule alone.

Accordingly a series of experiments was begun to determine if the presence of biogenic amine substrate altered the kinetics of stabilization by SAM, and if SAM altered the kinetics of substrate constituents. The results of a pilot experiment in which the effects of phenylethanolamine and SAM on PNMT thermal stability were tested showed that the degree of enzyme stabilization afforded by the combination of substrates was considerably greater than would be expected from either alone, or by the sum of their actions. These results suggested that the combination of substrate and SAM might stabilize the enzyme in a synergistic fashion.

This possibility was tested in a series of dose-response experiments, two of which are shown in Figure 2. S-adenosylmethionine alone resulted in a maximal stabilization of 1.95-fold, and the dose-response relationship seemed to follow the typical plateau curve. Phenylethanolamine stabilized the enzyme by only 1.2-fold, but the combination of phenylethanolamine and S-adenosylmethionine stabilized the enzyme by 4.31-fold. Moreover, the slope of the

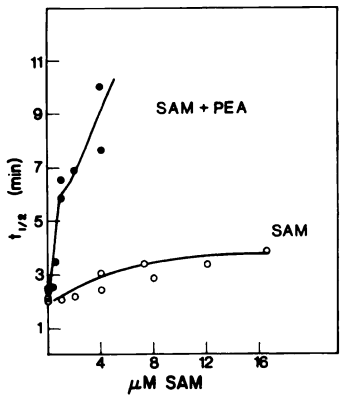


FIG. 2. *Effects of S-adenosylmethionine and phenylethanolamine on PNMT thermal stability*

Experiments were performed on purified bovine PNMT as described in METHODS. The results are derived from two experiments conducted on different days; data have not been corrected for assay variation. Various concentrations of S-adenosylmethionine were added to enzyme preparations in the presence or absence of phenylethanolamine; ([phenylethanolamine] = 1166 μM in experiment I, [phenylethanolamine] = 1041 μM in experiment II). The enzyme preparations were then subjected to thermal denaturation. Residual PNMT activity was determined, and the first-order rate constant of thermal denaturation determined (2-4). From k, the rate constant, the half-life was calculated by $t_{1/2} = \frac{\ln 2}{k}$. The values for $t_{1/2}$ were then plotted against S-adenosylmethionine concentration.

second curve suggested that phenylethanolamine markedly enhanced the affinity of SAM for PNMT. Because of the complex nature of this curve, values for the stabilization constant (K_s) (3) could not be computed with certainty.

Effects of phenylethanolamine and S-adenosylmethionine on PNMT tryptic stability. To confirm the findings described above, the synergistic effects of phenylethanolamine and SAM were tested in the tryptic degradation system. Previous results (3) had suggested that the tryptic degradation system was a more sensitive indicator of stabilization than the thermal denaturation system. Accordingly, a pilot experiment in which both phenylethanolamine and SAM were present was carried out. The results of this study showed that the combination of phenylethanolamine and SAM resulted in a marked degree of stabilization against tryptic proteolysis.

Phenylethanolamine stabilized PNMT by 1.39-fold, SAM by 4.58-fold, and the combination of S-adenosylmethionine and phenylethanolamine stabilized the enzyme by 9.71-fold.

The results of this experiment were confirmed and extended by a series of dose-response studies (Fig. 3). These results suggest that the presence of phenylethanolamine and SAM exerts a profound effect on the stability of PNMT. The effect seen in the tryptic studies is similar to but much more dramatic than the results seen in the thermal denaturation studies. In the presence of phenylethanolamine, the SAM dose-response curve was shifted to the left, indicating that phenylethanolamine increased the affinity of *S*-adenosylmethionine for PNMT. In addition the curve

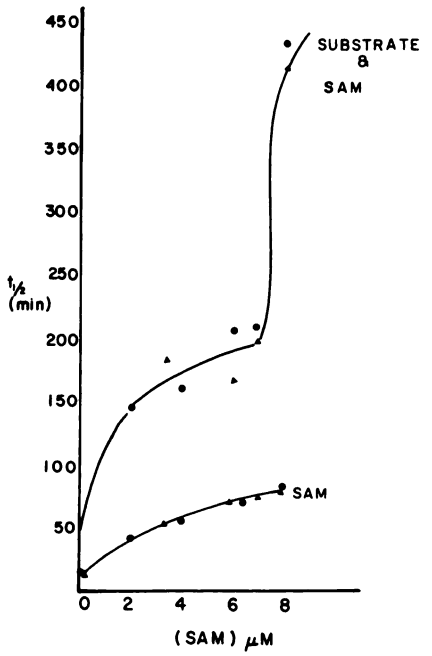


FIG. 3. *Effects of phenylethanolamine and S-adenosylmethionine on PNMT tryptic stability*

Various concentrations of SAM were added to enzyme mixtures, as described above and in (2, 3), with and without 1166 μM phenylethanolamine. The various preparations were subjected to tryptic proteolysis. Samples were then assayed for residual PNMT activity. Regression coefficients and half-lives were determined according to methods described in (2, 3), and half-lives were plotted against [S-adenosylmethionine]. ▲, Experiment I; ●, Experiment II.

showed a complex profile, with a marked increase in stabilization occurring at the end of a plateau. Because of the complex nature of the curve, no attempt was made to calculate K_a values by the double-reciprocal method previously described (3).

Effects of norepinephrine and S-adenosylmethionine on PNMT tryptic stability. Our previous studies, as well as those described above, had shown that phenylethanolamine was an effective stabilizer of PNMT against both thermal and tryptic degradation. Norepinephrine, the natural PNMT substrate, on the other hand, was ineffective as a stabilizer against thermal degradation, and only weakly effective as a stabilizer against tryptic degradation. The possibility existed, therefore, that our findings with phenylethanolamine, while of interest, had little or no significance *in vivo*. Accordingly, a series of studies testing the efficacy of norepinephrine, the natural PNMT substrate, as a facilitator of SAM stabilization were undertaken.

In a pilot experiment the combination of norepinephrine and SAM resulted in a marked degree of PNMT stabilization against tryptic degradation. Norepinephrine alone stabilized the enzyme by 1.73-fold, while SAM stabilized by 7.82-fold. The combination of the two compounds stabilized the enzyme by 21-fold.

A series of SAM dose-response studies, carried out in the presence or absence of norepinephrine, indicated that the catecholamine markedly facilitated the stabilizing properties of SAM. The results of three such studies are shown in Figure 4. S-adenosylmethionine stabilization in the absence of norepinephrine shows the fairly typical hyperbolic profile. Norepinephrine appears to have effects similar to those of phenylethanolamine. In the presence of norepinephrine the affinity of SAM for PNMT is enhanced. Moreover the stabilization properties of SAM and norepinephrine show a second rising phase after reaching an initial plateau. The plateau phase is not as pronounced as that seen in the phenylethanolamine curve (Fig. 3).

Effects of S-adenosylmethionine on the norepinephrine dose-response stabilization curve. Our previous studies were done

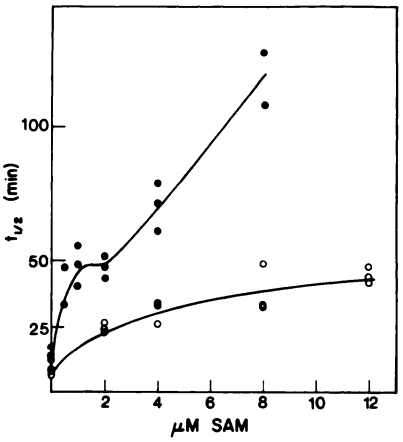


FIG. 4. *Effects of norepinephrine and S-adenosylmethionine on PNMT tryptic stability*

Various amounts of SAM were added to enzyme preparations as described above and in (2, 3), with or without norepinephrine (19.7 μM). The samples were then subjected to proteolysis by trypsin (1 μg/sample) for 0, 5, 10, and 20 min, then assayed for residual PNMT activity. Regression coefficients and half-lives were determined, according to methods described in (2, 3), and half-life was plotted against [S-adenosylmethionine].

testing the effect of a fixed concentration of substrate and a varying concentration of SAM. These results indicated that the presence of substrate both enhanced the stabilizing properties of SAM, and increased the affinity of this compound for PNMT.

The possibility existed, therefore, that SAM and substrate might be mutually synergistic. We next considered the possibility that the presence of SAM might influence the dose-response curve for norepinephrine. Accordingly a series of experiments was designed to test this. The results of two such experiments are shown in Figure 5. In contrast to the previous studies, where norepinephrine markedly enhanced the affinity of SAM for PNMT, SAM had no effect on the norepinephrine dose-response curve. SAM alone stabilized the enzyme by 2.33-fold, norepinephrine alone stabilized by about 3-fold, and the combination by 6.67-fold. Although the combination of norepinephrine and SAM was markedly more effective than either agent alone, there seemed to be no shift in the norepinephrine dose-response curve. Moreover the second rapidly-rising portion of curve seen in Figures 3 and 4 is not seen as dramatically in Figure 5. It thus appeared that SAM did not greatly facilitate norepinephrine stabilization of the enzyme, despite the finding that norepinephrine markedly facilitated SAM stabilization.

Mathematics of stabilization of PNMT by SAM and substrate. A complex relationship exists between substrate and SAM on PNMT stabilization against degradation. In every experiment in which the SAM dose-response effects on enzyme stability were determined in the presence of substrate, a marked enhancement of stabilization was seen. The extent of this stabilization was far greater than would be expected from the sum of the stabilization values of substrate alone or SAM alone. Similarly, the product of these values was much less than the observed degree of stabilization.

In an effort to develop a formula for the extent of stabilization seen in the presence of substrate and SAM, a number of combinations were tested. The formula that most closely fit the observed data was the following:

$$S_c = S_{SAM} + S_{NE} + (S_{SAM} \cdot S_{NE})$$

where S_c = calculated stabilization; S_{SAM} = stabilization by SAM alone; S_{NE} = stabilization by norepinephrine alone. This relationship was derived empirically but

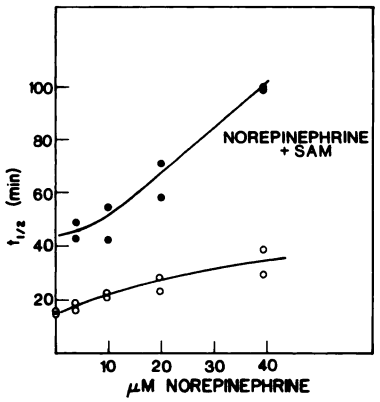


FIG. 5. *Effect of SAM on the tryptic stabilization by norepinephrine*

S-adenosylmethionine was present at a concentration of 4 μM (top curve) or omitted from the incubation mixture. The concentration of norepinephrine was varied, and at each concentration the half-life of PNMT in trypsin was determined.

seemed to be reasonably accurate in describing the observed degree of stabilization. In a typical stabilization experiment, for example, in which phenylethanolamine was present in fixed concentration but the concentration of S-adenosylmethionine was varied, the maximal stabilization seen by SAM alone was 1.67-fold, the stabilization by phenylethanolamine alone was 1.32-fold and the combination of substrate and SAM stabilized the enzyme by 5.12-fold. The formula above would have predicted a 5.19-fold degree of stabilization. In many cases, this degree of similarity was obtained; in others observed and calculated stabilization values diverged.

To test whether the empirical derivation was, in fact, a valid estimate of the data, the results of 11 SAM dose-response studies in which substrate was present were examined. The observed degree of stabilization in the presence of SAM and substrate were compared with the estimates derived by the formula above. The observed stabilization was then plotted against the calculated stabilization estimated from the formula, and correlation analysis carried out. The results are shown in Figure 6. The data show a striking correlation between the degree of stabilization actually observed and that calculated from the empirically-derived formula.

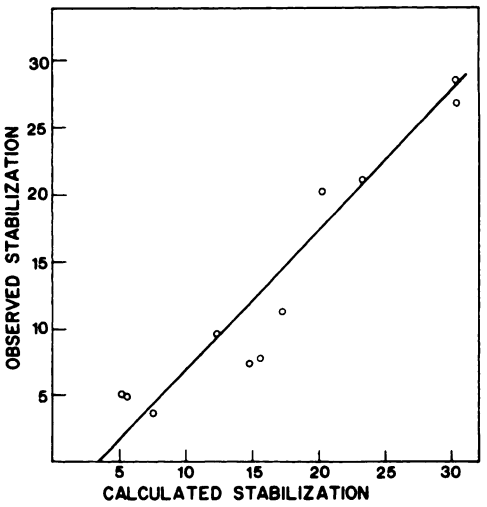


FIG. 6. *Comparison of observed stabilization and calculated stabilization of SAM-substrate studies*

We observed that in most experiments the observed stabilization closely approximated the stabilization calculated by the formula $S_c = S_s + S_{SAM} + (S_s \cdot S_{SAM})$. To determine the validity of this formula, the calculated stabilization for 11 dose-response experiments was plotted against the stabilization observed in those experiments. If a perfect correlation existed, then regression analysis should have given the line with slope of 1.00, which passed through the origin. The actual line was given by $Y = -3.49 + 1.05x$; $r_p^2 = 0.90$, ($p < 0.0001$). Thus the actual line deviated somewhat from the ideal line. The coefficients of the calculated line were: Y intercept = -3.49 ± 2.14 and slope = 1.05 ± 0.12. These did not differ significantly from Y intercept = 0 and slope = 1 respectively.

Model for the regulation of PNMT proteolysis by substrate and by S-adenosylmethionine. The findings described in this report suggested the following model, which is summarized in Figure 7. The PNMT molecule is conceptualized as having a number of proteolytically vulnerable sites in the regions of the norepinephrine (substrate) and SAM binding sites. In Figure 7A, three such sites that are vulnerable to proteolytic cleavage are hypothesized.

The binding of norepinephrine (Fig. 7B) results in a conformational shift in the PNMT molecule such that the proteolytically vulnerable site (No. 1) in this region is protected from degradation. The other sites (2 and 3) remain vulnerable; the effect of binding substrate alone is about a two-fold stabilization of the enzyme. Binding of SAM, on the other hand (Fig. 7C), results in an enzyme conformational shift such that site 3, but not sites 1 and 2 are protected. This confers an approximately six-fold degree of stabilization, as measured in several experiments.

When both norepinephrine and SAM are bound (Fig. 7D), maximum stabilization of PNMT against proteolysis is achieved. Not only are sites 1 and 3 protected, but the apposition of substrate and SAM induces a second conformational shift such that site 2 is now protected, and the enzyme is maximally stabilized against proteolysis. Under these conditions, PNMT degradation by trypsin in the procedures we routinely use is very difficult to demonstrate. At times half-lives for PNMT in trypsin as long as 450 min (control = 10–15 min) have been measured.

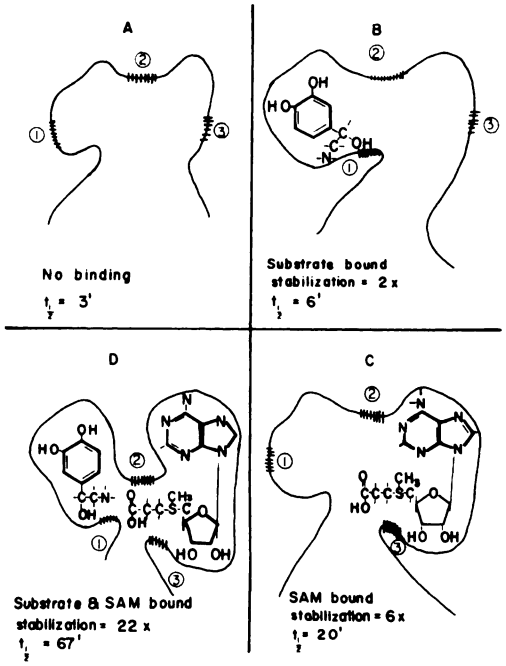


FIG. 7. *Model for the regulation of PNMT proteolysis by SAM and substrate*
Details are in the text.

DISCUSSION

The results of the reports published in this series demonstrate that glucocorticoid hormones regulate the steady-state levels of PNMT by preventing the intracellular proteolysis of the enzyme. While the precise mechanism of the glucocorticoid effect is not entirely worked out, the following points have been verified.

1. Hypophysectomy reduces intracellular PNMT levels by accelerating the *in vivo* proteolysis of the enzyme. When rats are hypophysectomized, immuno-titratable PNMT levels fall markedly, and are restored by dexamethasone treatment. By combined pulse-labeling and immunochemical isolation procedures, we determined that PNMT levels declined after hypophysectomy because the enzyme was being degraded more rapidly *in vivo*; this process was reversed by dexamethasone administration (1).

2. Concomitant with the acceleration in *in vivo* degradation, hypophysectomy caused an increased vulnerability of PNMT to thermal and tryptic degradation. PNMT from hypophysectomized rats does not differ in its electrophoretic mobility (2), its K_m for phenylethanolamine or SAM (1), or its immunogenicity (1). However, PNMT in adrenal supernatants of hypophysectomized rats is much more rapidly degraded by heating at 50°, or by tryptic proteolysis (2).

3. The thermal and proteolytic stability of PNMT is controled by an endogenous adrenal stabilizing factor. This compound is present in the adrenals of normal rats, and is lost after hypophysectomy. When added to supernatant preparations from hypophysectomized rats, this stabilizing factor restores the thermal and tryptic stability of PNMT. Glucocorticoid or ACTH administration to hypophysectomized rats restores stabilizing factor levels, restores the thermal stability of the enzyme, and prevents its *in vivo* proteolysis (1). The endogenous adrenal stabilizing factor is a freeze-thaw labile, dialyzable small molecule which binds directly to PNMT. A purified preparation of the stabilizing factor had ultraviolet absorption and paper chromatographic properties similar to those of SAM, the methyl donor in the PNMT reaction (3).

4. Addition of varying concentrations of SAM to adrenal preparations from hypophysectomized rats resulted in marked stabilization of the enzyme against either thermal or tryptic degradation (3). Administration of SAM to hypophysectomized rats resulted in partial restoration of PNMT levels and of the thermal stability of the enzyme (3). Thus SAM acted both *in vivo* and *in vitro* as a stabilizing agent. Dexamethasone, in contrast, was ineffective in stabilizing PNMT against *in vitro* degradation (2). SAH, the demethylated analogue of SAM, was ineffective in stabilizing PNMT against thermal degradation, thus ruling out this compound as the "endogenous stabilizing factor." However, SAH was effective in stabilizing PNMT against tryptic degradation, thus eliminating the possibility that SAM stabilized PNMT by methylating free carboxyl groups on the PNMT molecule (3).

5. Estimates of the K_s (stabilization constant) values for SAM (0.5 μM) were below

the K_m of SAM for PNMT (2.8 μM), but higher than the endogenous concentrations of SAM in our sample preparations (0.3 μM). This suggested the existence of another compound that either facilitated the action of SAM or was an original stabilizing factor. Furthermore, if SAM could stabilize PNMT against degradation, it seemed likely that other constituents of the PNMT reaction, such as substrate or product, could function in a similar manner. Accordingly, both norepinephrine, the natural substrate for PNMT, and epinephrine, the reaction product, were tested for stabilizing ability. Epinephrine had no stabilizing effect on either thermal or tryptic degradation of PNMT. Norepinephrine was slightly effective as a stabilizer against tryptic degradation, but ineffective as a stabilizer against thermal degradation. In testing a variety of PNMT substrates for stabilizing efficacy, the most effective was phenylethanolamine, and the least effective was norepinephrine. These results suggested that substrate by itself probably played a limited direct role in stabilizing PNMT against degradation, a role which was secondary in importance to that played by S-adenosylmethionine.

6. Although substrate did not play the major role in stabilizing PNMT against proteolysis, the presence of norepinephrine or phenylethanolamine markedly augmented the stabilizing action of SAM. Moreover, the presence of substrate greatly increased the affinity of SAM for PNMT, thus making the low endogenous SAM concentrations present in our sample preparations highly effective in stabilizing the enzyme against *in vitro* degradation.

These findings therefore suggest that the binding of substrates and cofactors may play an important role in regulating *in vitro* enzyme degradation. The extension of this possible role to intracellular function should be undertaken with caution. The following points require further experimental verification before the data obtained *in vitro* can be taken as evidence of an intracellular regulatory mechanism: 1) SAM levels must be shown to be under glucocorticoid control and 2) the administration of SAM to hypophysectomized rats should prevent the increased degradation of PNMT caused by hypophysectomy.

In support of the first proposition, recent evidence from our laboratory shows that hypophysectomy causes a marked reduction in adrenal SAM content (Wong, D. L. and Ciaranello, R. D. in preparation). The reduction in SAM content was commensurate with the decline in PNMT levels observed in the same animals. The second hypothesis, that SAM administration slows PNMT degradation *in vivo*, is currently being investigated in our laboratory.

With the caveat that proof that our *in vitro* findings describe an *in vivo* regulatory mechanism is not yet complete, we can nonetheless ask two relevant questions: 1) what is the evidence that similar regulatory mechanisms exist for the control of the proteolysis of other enzymes, and 2) can our findings be generalized to other SAM-dependent methyltransferases?

There is considerable evidence that the model we presented of ligand-induced conformational shifts affecting enzyme proteolytic vulnerability is a valid one. Katunuma et al. (10) have shown that the proteolysis of the pyridoxal-dependent rat intestinal mucosal enzymes is carried out by a single proteolytic enzyme. This protease rapidly degrades the apo-enzyme, but the pyridoxal phosphate-containing holoenzyme is resistant to proteolytic attack. Recently Dunaway et al. (11) have demonstrated the existence of a peptide stabilizing factor that is regulated by insulin and that controls the degradation of phosphofructokinase (PFK-L_2). Although its mechanism is unknown, this stabilizing factor is thought to alter the susceptibility of PFK-L_2 by controling the susceptibility of the enzyme to degradation. Similarly, Schimke has proposed a model whereby presence of substrate alters the tertiary conformation of an enzyme, making it more resistant to proteolytic destruction (12). Matsuzawa (13) has proposed a reversible conversion between the holoenzyme and apo-enzyme forms of ornithine δ-aminotransferase. When pyridoxal phosphate is bound, the holoenzyme assumes a stable, "tight-state" configuration, whereas the apo-enzyme is in a "relaxed" conformation that is labile to

proteolytic degradation. Lastly, there seems to be general agreement that data derived from *in vitro* systems using model proteases such as trypsin and pronase accurately reflect the degradation of enzymes by intracellular proteases. The sort of model we are currently proposing seems to be one that has already gained acceptance among other workers studying different enzyme systems.

It is also important to consider whether our findings are generalizable to other methyl transfer reactions, including biogenic amine synthesis and inactivation, tRNA methylation, protein methylation, phospholipid methylation, and steroid methylation. In a preliminary effort to assess this, we have recently found that hydroxyindole O-methyltransferase, the pineal enzyme that catalyzes the terminal step in melatonin biosynthesis, seems to follow the same pattern we have observed for PNMT. After hypophysectomy, pineal HIOMT levels fall profoundly, and can be restored by glucocorticoid or SAM administration. The presence of SAM markedly stabilizes HIOMT against tryptic attack, while N-acetylserotonin, the enzyme substrate, is relatively ineffective in this role. The presence of substrate, however, facilitates the stabilizing efficacy of SAM (Sandrock, A., Leblanc, G., and Ciaranello, R. D. Manuscript in preparation).

Our data suggest, then, that factors such as ligand binding that affect the tertiary conformation of an enzyme may play a critical role in determining the rate at which the enzyme is degraded by intracellular proteases. If true, this would explain the apparent "specificity" of proteolytic reactions. One of the more troublesome questions plaguing investigators in this field has been the issue of "specificity" of proteases. If proteases are specific for their enzyme substrates, then each enzyme, or class of enzymes, would have its own protease. Furthermore, because proteases themselves must undergo intracellular destruction, does this mean each protease has its own protease? If so, much of the cell enzymatic machinery must be given over to "garbage" functions. This seems hardly a parsimonious utilization of cellular resources.

The models we and others have presented avoid this troublesome postulate. By the reasoning we are proposing a single protease might regulate the turnover of many enzymes. Association of those enzymes with substrates, cofactors or other ligands would determine the enzyme conformation, and thus its proteolytic vulnerability. Proteolytic specificity, then, would reside in the interaction between the enzyme and its ligand. The data from several laboratories would seem to support this argument. Moreover, our recent findings that SAM might affect HIOMT degradation as well as the proteolysis of PNMT would offer further credence to this viewpoint.

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