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S-Adenosylmethionine-dependent Protein Methylation in Mammalian Cytosol via Tyrphostin Modification by Catechol-*O*-methyltransferase*

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It has previously been shown that incubation of mammalian cell cytosolic extracts with the protein kinase inhibitor tyrphostin A25 results in enhanced transfer of methyl groups from *S*-adenosyl-[*methyl*-^{3}H]methionine to proteins. These findings were interpreted as demonstrating tyrphostin stimulation of a novel type of protein carboxyl methyltransferase. We find here, however, that tyrphostin A25 addition to mouse heart cytosol incubated with *S*-adenosyl-[*methyl*-^{3}H]methionine or *S*-adenosyl-[*methyl*-^{14}C]methionine stimulates the labeling of small molecules in addition to proteins. Base treatment of both protein and small molecule fractions releases volatile radioactivity, suggesting labile ester-like linkages of the labeled methyl group. Production of both the base-volatile product and labeled protein occurs with tyrphostins A25, A47, and A51, but not with thirteen other tyrphostin family members. These active tyrphostins all contain a catechol moiety and are good substrates for recombinant and endogenous catechol-*O*-methyltransferase. Inhibition of catechol-*O*-methyltransferase activity with tyrphostin AG1288 prevents both base-volatile product formation and protein labeling from methyl-labeled *S*-adenosylmethionine in heart, kidney, and liver, but not in testes or brain extracts. These results suggest that the incorporation of methyl groups into protein follows a complex pathway initiated by the methylation of select tyrphostins by endogenous catechol-*O*-methyltransferase. We suggest that the methylated tyrphostins are further modified in the cell extract and covalently attached to cellular proteins. The presence of endogenous catechols in cells suggests that similar reactions can also occur *in vivo*.

S-Adenosylmethionine-dependent protein methylation is a common post-translational modification, occurring at N-terminal amino, arginine, lysine, modified aspartyl, and C-terminal carboxyl residues (1). These reactions not only create additional diversity in protein amino acids but also provide opportunities for biological regulation by reversible modifications (2–9).

We have been especially interested in identifying novel types of protein methylation reactions. In 1999, Bilodeau and Béliveau (10) demonstrated that *S*-adenosyl-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet)[2]-dependent protein methylation in rat kidney cytosolic extracts is greatly stimulated by the addition of the protein-tyrosine kinase inhibitor tyrphostin A25 and the protein phosphatase inhibitor sodium vanadate. These results suggested a regulatory interplay between protein phosphorylation and methylation systems. We were able to confirm these results in mouse kidney cytosol (11). We were also able to show that tyrphostin A25, in the presence or absence of vanadate, stimulates protein methylation in mouse heart cytosol, but not in testes or brain cytosol (11). Of a variety of tyrphostins tested only the A25 and A47 derivatives were active in stimulating protein methylation in kidney cytosol. Analysis of a ^{3}H-methylated 15-kDa polypeptide indicated that the radioactivity was not due to protein lysine or arginine methylation. The radioactivity was volatilized after base treatment, suggesting that the modification is a type of carboxyl methylation, but is not due to the known protein L-isoaspartyl methyltransferase or C-terminal carboxyl methyltransferases (11). These results suggested that tyrphostin A25 could stimulate a novel signaling pathway involving methylation. However, the identity of the methylated substrate and methyltransferase remained elusive.

The tyrphostins are a family of synthetic small molecule inhibitors of protein-tyrosine kinases based on the naturally occurring inhibitor erbstatin (12, 13). Due to the importance of kinases in many cellular signaling pathways, including those leading to cell proliferation and cancer, the tyrphostins have been extensively studied as possible therapeutic agents (12, 14, 15). Tyrphostins have also been shown to have inhibitory activities toward adenylate cyclase (16) and a variety of GTP-utilizing enzymes (17). The significant up-regulation of methylation by tyrphostin A25 may thus be highlighting new roles of methylation in signal transduction pathways.

We sought to further understand tyrphostin-stimulated methylation in mouse heart cytosol by identifying the methyl-accepting proteins and the specific methyltransferases involved. In the course of this work, we discovered that the methyltransferase responsible is catechol *O*-methyltransferase (COMT) acting on the tyrphostin molecule itself. We show

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[2] The abbreviations used are: [^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]methionine; [^{14}C]AdoMet, *S*-adenosyl-[*methyl*-^{14}C]methionine; COMT, catechol-*O*-methyltransferase.

here that a cellular modification of the methylated species can then be covalently attached to various cytosolic polypeptides mimicking a typical protein methylation reaction.

Although catechol-protein adducts are known in the literature, the addition of methylated catechols has not been previously observed (18–21). The reactions described here may represent a novel cellular pathway for methylated catechols.

EXPERIMENTAL PROCEDURES

Chemicals—All tyrphostins were obtained from Calbiochem and dissolved in Me_2SO to a stock concentration of 1 mM. Catechol (pyrocatechol, 1,2-benzenediol) was obtained from Sigma-Aldrich and brought up in water to a stock concentration of 1 mM.

Cytosolic Extract Preparation and Methylation Assay—Wild-type mice were used that had a genetic background of ~50% 129svJae and 50% C57BL/6 (11). Mice were weaned at 20 days and maintained on a NIH-31 Modified Mouse/Rat Diet #7013 on a 12-h dark/light cycle. 250 mM sucrose, 5 mM HEPES buffer (adjusted to pH 7.2 with Tris base) at 0 °C was added to harvested organs (5 ml/g wet weight). Homogenates were made in the presence of 1 mM phenylmethylsulfonyl fluoride and the recommended concentration of Complete Protease Inhibitor Cocktail tablets (Roche Applied Science) using a tight-fitting Teflon pestle rotating at 310 rpm for 1 min. Lysates (0.5–1 ml) were then centrifuged at 13,000 × *g* for 50 min in 1.5-ml plastic microcentrifuge tubes, and supernatants were collected and stored in aliquots at −80 °C. Protein concentrations (5–20 mg/ml) were determined after precipitation with 10% trichloroacetic acid using the Lowry method with bovine serum albumin standards.

Supernatants of the tissue lysate (30 or 60 μg of protein) were incubated for 60 min at 37 °C in a total volume of 30 μl with either 1 μl of 0.30 mM *S*-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C]AdoMet, Amersham Biosciences, 50–60 mCi/mmol, in dilute sulfuric acid, pH 2.5–3.5) or 3 μ1 of 12 μM [^{3}H]AdoMet (Amersham Biosciences, 70–81 Ci/mmol, in dilute HCl:ethanol (9:1), pH 2–2.5) in 150 mM HEPES buffer adjusted to pH 7.5 with Tris base. Tyrphostins (in Me_2SO) were added when indicated to a final concentration of 100 μM (10% Me_2SO final concentration). For controls, Me_2SO was used at 10% final concentration.

SDS-PAGE—An equal volume of SDS gel sample buffer (180 mM Tris-HCl, pH 6.8, 4% SDS, 0.1% β-mercaptoethanol, 20% glycerol, and 0.002% bromphenol blue) was added to samples, which were then heated at 100 °C for 3 min. Samples were then electrophoresed for 14 h at 8 mA followed by 4 h at 12 mA on a 1.5-mm thick gel containing 4.3% acrylamide, 0.15% *N*,*N*-methylenebisacrylamide in a 2-cm stacking gel and 12.1% acrylamide, 0.42% *N*,*N*-methylenebisacrylamide in a 10.5-cm resolving gel. Gels were stained using Coomassie Brilliant Blue R-250 for 24 h and de-stained with 10% methanol, 5% acetic acid for 48 h. After a photograph was taken, gels were treated with EN^{3}HANCE (PerkinElmer Life Sciences) for 1 h and rinsed in water for 20 min. Gels were dried at 80 °C for 2 h under vacuum and exposed to Kodak BioMax MS film at −80 °C to obtain fluorographs.

Vapor-diffusion Assay for Base-labile Methyl Groups—An assay for quantitating radiolabeled methyl groups in ester linkages has been developed based on the detection of volatile radioactivity (as methanol) after ester hydrolysis by base treatment (22–24). This assay will also detect methyl groups in non-ester linkages where base treatment results in methanol or other volatile derivatives of the methyl group. For a typical 30-μl reaction mixture, 2 μl is first taken for determination of total radioactivity with 5 ml of Safety-Solve Complete Counting Mixture (Research Products International, Mt. Prospect, IL). NaOH (28 μl of a 2 N solution) was then added to the remaining 28 μl of the reaction mixture. A total of 40 μl of this mixture was then spotted onto an accordion-folded strip (8.5 cm by 3.5 cm) of thick filter paper (Bio-Rad 1650962) and wedged into the neck of a 20-ml vial containing 5 ml of scintillation Fluor. After capping, the samples were incubated for 2 h at room temperature. The filter paper, containing the non-volatile radioactive components, was then removed, and the vials were recapped and counted in a Beckman LS6500 scintillation counter.

COMT Assay—Recombinant porcine liver COMT was obtained from Sigma-Aldrich and brought up in 1% bovine serum albumin to a stock concentration of 0.01 unit/μl (one unit produces 1 nmol of total *O*-methylated products from 3,4-dihydroxyacetophenone per hour at pH 7.6, 37 °C). This enzyme was assayed in a final volume of 50 μl by modifications of previously described methods (25, 26). Assay mixtures contained COMT at 0.001 unit/μl in a buffer of 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 500 μM dithiothreitol with 1.25 μM [^{3}H]AdoMet and 100 μM of methyl-accepting substrate. After 30 min at 37 °C, 50 μl of water was added followed by 100 μl of 2 N HCl and 200 μl of ethyl acetate. Samples were then vortexed and centrifuged at 17,900 × *g* for 5 min. Finally, 100 μl of the top ethyl acetate layer was mixed with 5 ml of scintillation fluid and counted for the ^{3}H-methylated catechol product.

RESULTS

Previous studies suggested that tyrphostin A25 greatly stimulates a potentially novel protein methylation reaction when added to mouse heart cytosolic extracts in the presence of [^{3}H]AdoMet (11). To identify and characterize the methylated polypeptides, we first compared the fractionation of ^{3}H-labeled heart cytosolic proteins by size-exclusion chromatography for samples incubated with and without tyrphostin A25 (Fig. 1). When fractions were analyzed by total radioactivity, we found a large increase with tyrphostin in the labeling of protein fractions in the void volume eluting at 125 min and in the included volume eluting from 200 to 300 min (Fig. 1*A* and *inset*). Peaks corresponding to unreacted [^{3}H]AdoMet were found as expected in the total volume eluting at ~330–450 min. However, we were surprised to see a relatively large peak of radioactivity eluting between 510 and 570 min, after the total volume, suggesting an interaction of this material with the column resin. Significantly, this material was only detected in the tyrphostin-containing sample. To attempt to identify this material, we applied tyrphostin A25 to the column and observed (by its orange color) that it was adsorbed to the resin and was not eluted even in two column volumes of buffer. This result sug-

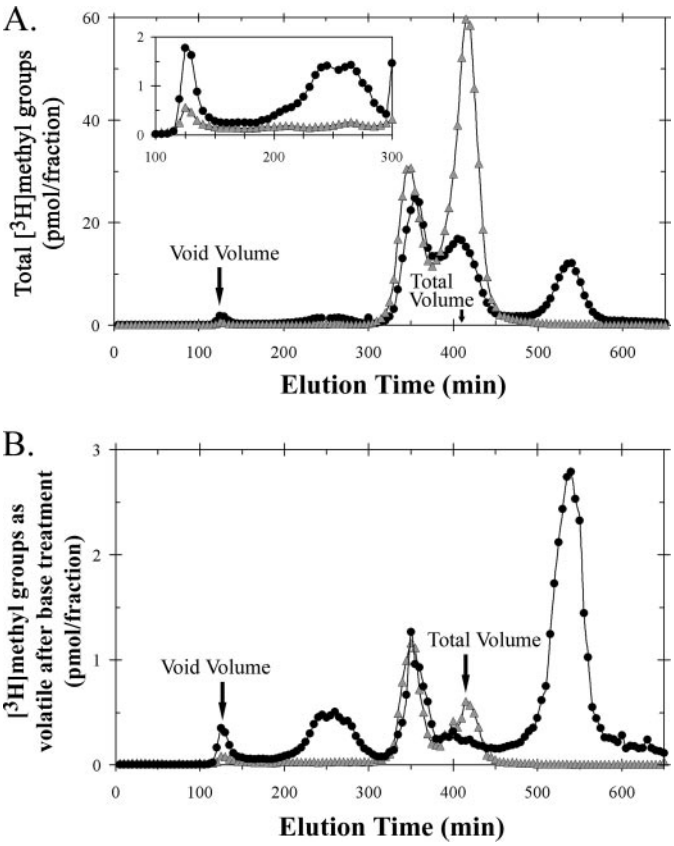


FIGURE 1. **Gel-filtration analysis of methylated products of mouse heart cytosol incubated with [^{3}H]AdoMet in the presence and absence of tyrphostin A25.** Cytosol (1.5 mg protein) was incubated in a total volume of 0.75 ml with 1.3 μM [^{3}H]AdoMet (79 Ci/mmol) in 33 mM HEPES-Tris, pH 7.5, at 37 °C for 60 min in the presence or absence of 100 μM tyrphostin A25. Samples were then loaded on a Sephacryl S-300 size-exclusion column (1.5-cm inner diameter, 91-cm bed height, and 162-ml volume) equilibrated with 25 mM HEPES-Tris buffer, pH 7.5, at 4 °C. The column was eluted in the same buffer at a flow rate of 0.4 ml/min, and 5-min fractions were collected. Aliquots of each fraction (0.5 ml) were analyzed for total radioactivity after counting in 5 ml of scintillation fluid; ^{3}H-methyl groups were calculated from the specific radioactivity (*A*). Aliquots of each fraction (50 μl) were combined with an equal volume of 2 N NaOH and analyzed by the vapor-diffusion assay as described under "Experimental Procedures" to determine the number of base-labile ^{3}H-methyl groups (*B*). Results for the sample containing tyrphostin A25 are shown in *circles* (●); those for the sample without tyrphostin A25 are shown in *triangles* (▲). The positions of the void and total volume are shown with *arrows*; the *inset* in *A* shows a magnification of the region between 100 and 300 min.

gested that the late eluting peak at 510–570 min in the tyrphostin sample of Fig. 1*A* may be a modified form of tyrphostin.

Previous analysis of tyrphostin-stimulated protein methylation suggested that the ^{3}H-methyl groups were incorporated as methyl esters, because they could be detected as a volatile species, presumably [^{3}H]methanol, following base hydrolysis (11). We thus further analyzed the gel-filtration fractions for base-labile volatility (Fig. 1*B*). We found that a significant fraction (40%) of the radioactivity in the protein fractions eluting between 200 and 300 min was volatilized under these conditions. We also showed that ~25% of the radioactivity in the late peak at 510–570 min could be volatilized after base treatment. These results suggest that the methylation stimulated by tyrphostin may be complex and that the products may include both proteins and tyrphostin or its derivatives. Significantly, we found that the total volatile radioactivity produced in all of the fractions of the tyrphostin-treated sample was much greater than that of the control sample (Fig. 1*B*). We thus realized that methylation stimulated by tyrphostin A25 could be assayed directly by analyzing the total production of base volatile radioactivity in a single reaction mixture.

Following these results, we developed an assay where the reaction of cytosolic extracts, [^{14}C]AdoMet, and tyrphostin could be monitored by detecting volatile radioactivity after base treatment in a vapor-diffusion assay. We validated this assay to show that it reflected the previously seen tyrphostin A25-stimulated formation of methylated proteins. We first asked whether the specificity for different tyrphostin derivatives seen in protein methylation (11) would be replicated in this vapor-diffusion assay. In Table 1, we show that stimulation of methylation is clearly seen in heart cytosol with tyrphostin A25 and A47, two derivatives previously shown to stimulate protein methylation in kidney cytosol (11). Of the compounds previously shown to be inactive, we also found little or no stimulation in the vapor-diffusion assay (11) (see Table 1). We then tested additional tyrphostin derivatives with structural similarity to the active tyrphostins A25 and A47. We found that A51 was even more active than A25, but little or no activity was found for AG30 and B40 (Table 1).

Analysis of the structures of the most active tyrphostin derivatives suggest the importance of three hydroxyl groups on the aromatic ring present in A25 and A51. It is unclear, however, why A47 is more active than A23 or related compounds that also have two ring hydroxyl groups.

We then asked if the specific tyrphostin dependence of protein labeling in heart cytosol corresponded to the results of the vapor-diffusion assay shown in Table 1. Using an SDS-PAGE assay, we observed only tyrphostins A25, A47, and A51 showed an increase in labeled peptide bands, consistent with the results of the vapor-diffusion assay (Fig. 2, *bottom panel*). Additionally, we observed a significant amount of radioactivity midway in the stacking gel of the tyrphostin A25 and A51 samples, suggesting the formation of labeled protein aggregates. Aggregated species can also be seen in the Coomassie-stained gel at the interface of the stacking and running gel for the A25 and A51 samples (Fig. 2, *top panel*). Smaller amounts of radiolabeled aggregates at these positions are also seen with the B tyrphostins.

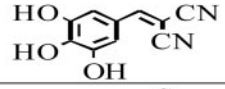
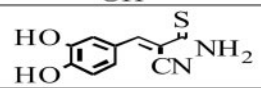
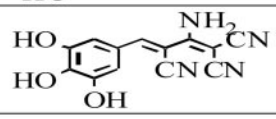
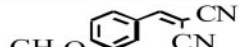
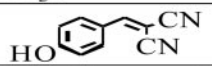
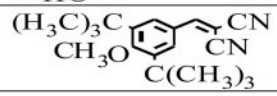
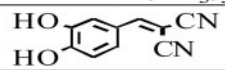
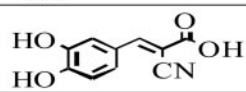
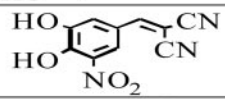
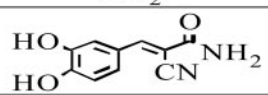
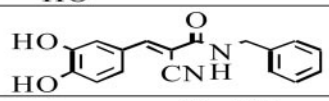
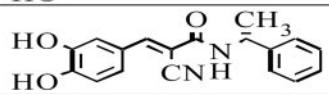
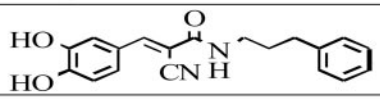
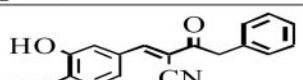
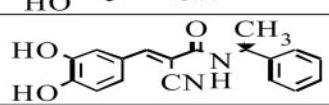
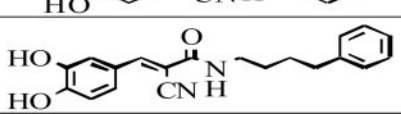
The results in Fig. 1 suggested the possibility that tyrphostin could stimulate small molecule as well as protein methylation. We thus paired the vapor-diffusion assay with a separation of protein and small molecules in the heart cytosolic reaction mixtures by trichloroacetic acid precipitation (Fig. 3). This experiment showed that the bulk of the tyrphostin stimulation measured occurs in the trichloroacetic acid-soluble small molecule fraction (Fig. 3) and may represent the material eluting from 510 to 570 min in the gel-filtration analysis (Fig. 1).

Given these results, we asked whether the tyrphostin molecules themselves were being methylated. All of the active tyrphostins contained adjacent aromatic hydroxyl groups (Table 1) that may be recognized and methylated by cellular COMT. Accordingly, we analyzed the ability of the tyrphostin derivates in Table 1 to serve as substrates for recombinant porcine COMT. As shown in Table 1, all tyrphostins containing catechol (two adjacent aromatic ring hydroxyl groups) or pyrogallol

TABLE 1

Comparison of the effect of tyrphostin derivatives on methylation of heart cytosolic extracts with their activity as substrates for recombinant catechol-*O*-methyltransferase

The effect of tyrphostin A25 and its derivatives on base-volatile product formation in mouse heart cytosol extracts from [^{14}C]AdoMet was determined by the vapor diffusion assay as described under "Experimental Procedures" in the presence of 100 μM of the tyrphostin derivative or a control of the Me_2SO solvent alone. The ability of the tyrphostins to serve as methyl-accepting substrates of recombinant COMT was determined as described under "Experimental Procedures." Values are given as the average ± S.D. of at least triplicate samples.

Tyrphostin	Structure	Vapor Diffusion Methylation Activity (pmol/min/mg protein)	COMT Activity (pmol/min/unit enzyme)
DMSO control	-	8.6 ± 1.8	0.05 ± 0.01
A25		44.9 ± 7.8	1.65 ± 0.06
A47		24.8 ± 6.5	2.45 ± 0.10
A51		99.1 ± 6.8	1.79 ± 0.18
A1		9.1 ± 0.7	0.05 ± 0.01
A8		9.1 ± 1.2	0.05 ± 0.00
A9		9.6 ± 0.4	0.04 ± 0.01
A23		8.4 ± 0.7	2.44 ± 0.01
AG30		10.6 ± 0.2	3.67 ± 0.07
AG1288		9.1 ± 0.5	0.05 ± 0.01
B40		10.5 ± 0.9	2.98 ± 0.18
B42		11.3 ± 0.3	3.06 ± 0.10
B44		10.9 ± 0.4	3.06 ± 0.15
B46		11.4 ± 1.4	3.11 ± 0.24
B48		11.7 ± 0.7	3.06 ± 0.09
B50		12.8 ± 2.9	3.08 ± 0.11
B56		11.5 ± 0.4	2.84 ± 0.29

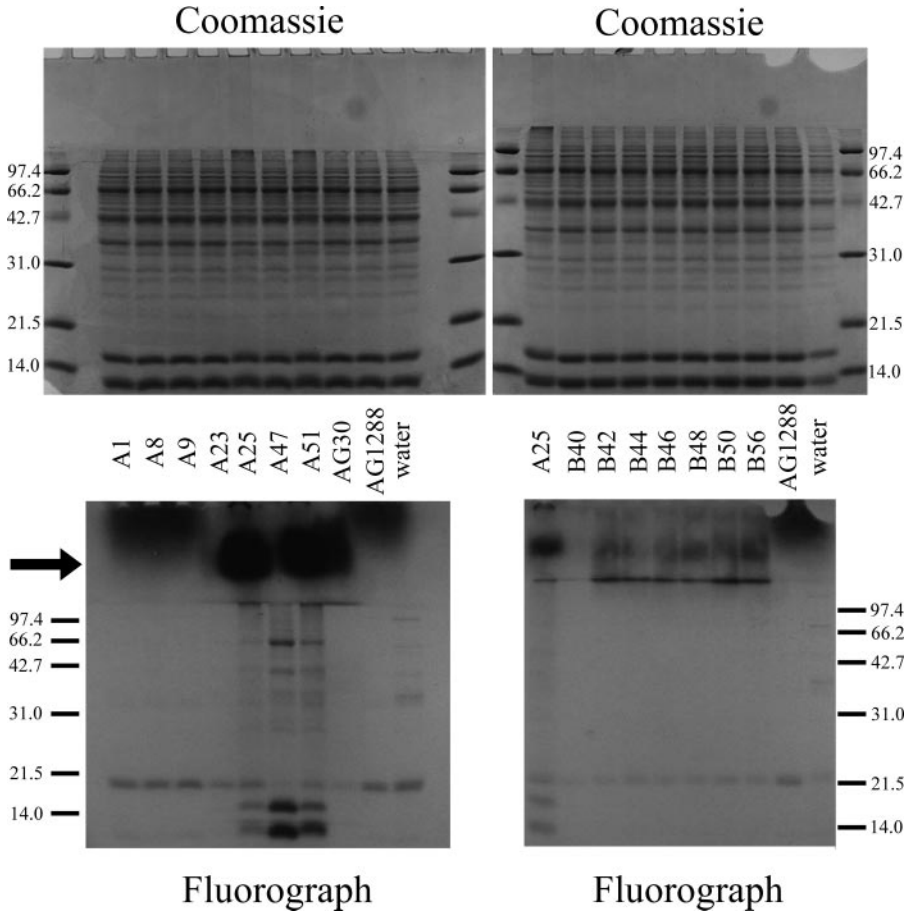


FIGURE 2. **SDS-PAGE analysis of methylated products of mouse heart cytosol incubated with [^{3}H]AdoMet in the presence and absence of tyrphostin derivatives.** Heart cytosol was incubated with [^{3}H]AdoMet and the indicated tyrphostin derivative at a concentration of 100 μM, and the polypeptides were analyzed by SDS-PAGE as described under "Experimental Procedures." Marker proteins in the gel outer lanes included phosphorylase, serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad low molecular weight standards). The Coomassie-stained gels are shown in the *upper panel*; fluorographs exposed for 2 months are shown in the *lower panel*. The *arrow* indicates polypeptide aggregates located within the stacking gel.

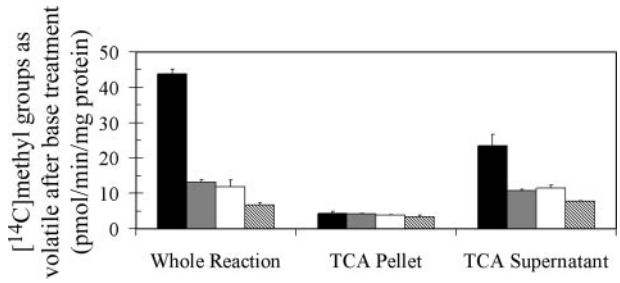


FIGURE 3. **Analysis by the vapor-diffusion assay of the methylated products of cytosol incubated with [^{14}C]AdoMet after trichloroacetic acid precipitation.** Mouse heart cytosol extracts were incubated in 30-μl reaction mixtures as described under "Experimental Procedures" in the presence of 100 μM tyrphostin A25 with either the addition of 10% Me_2SO (*black bars*) or 100 μM tyrphostin AG1288 (*light gray bars*). Controls are shown where cytosol was incubated without tyrphostins (*white bars*) or where buffer was used in place of cytosol in the presence of tyrphostin A25 (*lined bars*). Samples were either analyzed directly by the vapor-diffusion assay (whole reaction) or after trichloroacetic acid fractionation. Here, the reaction mixture was incubated with an equal volume of 20% trichloroacetic acid (*TCA*) and incubated at 20 °C for 10 min followed by centrifugation at 10,600 × *g* for 10 min. The supernatant was separated, mixed with 60 μl of 2 N NaOH, and 96 μl was taken for the vapor-diffusion assay. The pellet was resuspended in 50 μl of 1 N NaOH, and 40 μl was taken for the vapor-diffusion assay. The number of base-volatile ^{14}C-methyl groups was quantitated for duplicate experiments; the *bar* represents the deviation from the average.

(three adjacent aromatic ring hydroxyl groups) structures were found to be good substrates for COMT. In fact, only four or the compounds shown in Table 1 were not substrates for COMT. Tyrphostins A1, A8, and A9 do not have catechol or pyrogallol structures. Significantly, tyrphostin AG1288 contains a nitrocatechol structure that is known in other compounds to be associated with strong inhibition of COMT activity (27). This result suggests that tyrphostin AG1288 may also be an inhibitor of COMT activity. We tested this possibility directly and confirmed that AG1288 was an effective inhibitor of recombinant COMT with catechol (1,2-benzenediol) or with tyrphostin substrates (Fig. 4).

Our finding that tyrphostins A25, A47, and A51 are good COMT substrates and that AG1288 is an effective COMT inhibitor then provided us with an approach to determine if tyrphostin-stimulated methylation depends on COMT activity. We thus asked whether COMT inhibition in heart cytosol could inhibit the tyrphostin-dependent production of the base-volatile product. We found that COMT inhibition by AG1288 was sufficient to abolish the base-volatile product for all three active tyrphostin compounds (Fig. 5; see also Fig. 3). We then analyzed the effect of adding AG1288 to reaction mixtures analyzed by SDS-PAGE. As shown in Fig. 6, AG1288 prevented the tyrphostin A25-dependent methylation of heart and kidney cytosolic proteins, both as polypeptides within the running gel and as aggregated species in the stacking gel. These results indicate that the tyrphostin-stimulated methylation of both proteins and small molecules is dependent upon the activity of COMT, thus identifying the methyltransferase responsible for these modification reactions.

We confirmed the presence of endogenous COMT in mouse tissue cytosolic extracts and the ability of the endogenous enzyme to methylate tyrphostin A25. In Table 2, we show AG1288-inhibitable COMT activity in mouse heart, kidney, testes, and liver cytosol using both tyrphostin A25 and catechol (1,2-benzenediol) as substrates. These results suggest that these extracts are capable of modifying tyrphostin for possible protein attachment and methyl group base-labilization.

If this scenario is correct, we should be able to replicate the increased base-labile volatile radioactivity seen with [^{14}C]AdoMet, tyrphostin, and cytosol by simply adding ^{3}H-methylated tyrphostin A25 to the tissue extract. We thus

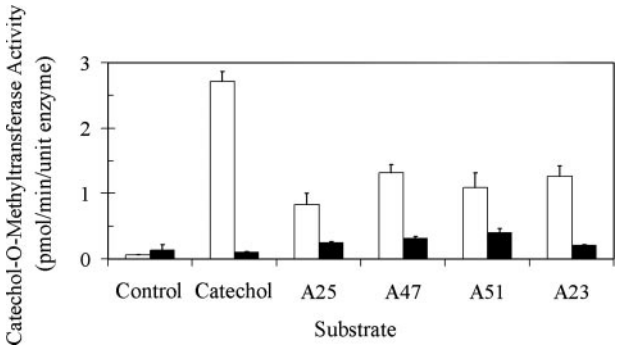


FIGURE 4. **Tyrphostin AG1288 inhibits recombinant COMT activity.** *In vitro* COMT assays were carried out as described under "Experimental Procedures" in the presence of 100 μM tyrphostin AG1288 (*black bars*) or 10% Me_2SO (*white bars*) using either no added methyl-accepting substrate (control, 10% Me_2SO), 100 μM catechol (1,2-benzenediol), or 100 μM of tyrphostin derivatives A25, A47, A51, or A23. Average values with the standard deviation are given from triplicate incubations.

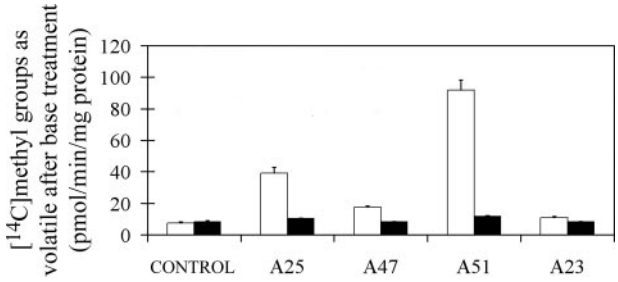


FIGURE 5. **Tyrphostin AG1288 inhibits ^{14}C-volatile product formation after base treatment of heart cytosol incubated with [^{14}C]AdoMet.** Mouse heart cytosol was incubated as described under "Experimental Procedures" in the presence of 100 μM tyrphostin AG1288 (*black bars*) or 10% Me_2SO (*white bars*) with the addition of 10% Me_2SO (control) or 100 μM of tyrphostin derivatives A25, A47, A51, or A23. Samples were analyzed by the vapor-diffusion assay. Average values with the standard deviation are given from triplicate incubations.

sought to bypass COMT involvement by using pre-methylated tyrphostin A25 as the sole radioactive methyl donor. As shown in Fig. 7*A*, significant stimulation of base-labile volatile radioactivity is seen in both heart and kidney cytosolic extracts with ^{3}H-methylated tyrphostin A25. In these experiments, we saw no effect of adding AG1288, confirming that the role of COMT was simply the initial methylation of tyrphostin. As a control, we show in Fig. 7*B* the expected dependence upon COMT activity when [^{14}C]AdoMet is used as a radiolabel donor.

We were then interested in asking how the methyl groups present as methyl ethers on tyrphostin became volatile after base treatment. We were able to demonstrate that the ^{3}H-methylated tyrphostin A25 product resulting from incubation with recombinant COMT is not base-volatile (data not shown). This result indicates that there are additional steps occurring in the tissue cytosolic extracts that lead to the labilization of the methyl group. A possible chemical scheme for these reactions based on known catechol chemistry is presented in Fig. 8 and discussed below.

In the experiments described so far, we have used heart and kidney cytosolic extracts to examine tyrphostin-stimulated methylation. We then wanted to see if similar reactions occurred in other tissues. In cytosolic extracts of brain, we observe no tyrphostin A25 stimulation of [^{3}H]- or [^{14}C]AdoMet-dependent methylation using SDS-PAGE analysis (Fig. 6) or the vapor-diffusion assay (Fig. 7*B*). However, when ^{3}H-methylated tyrphostin A25 was used in a vapor-diffusion assay, activity was seen (Fig. 7*A*). Given the near absence of COMT activity in brain cytosolic extracts (Table 2), these results suggest that methylation in brain may be similar to heart and kidney, but that the COMT required is found in the membrane rather than the cytosolic fraction (28, 29). A different situation was found in testes where we observed no tyrphostin stimulation of methylation in any of our assays (Figs. 6, 7*A*, and 7*B*), despite the fact that COMT activity was present (Table 2). Here, it appears that one or more factors responsible for subsequent steps leading to protein adduct formation and base-labile volatilization of the methyl group were lacking in testes. These results suggest that the full methylation pathway described here does not occur in all tissues.

The situation with tyrphostin-stimulated methylation in liver cytosol is more complex. In Fig. 6, using SDS-PAGE analysis, we showed that tyrphostin A25 can stimulate protein methylation in liver cytosol but that it is only partially inhibited by the AG1288 COMT inhibitor. ^{3}H-Methylated tyrphostin A25 could stimulate a moderate production of base-labile volatile radioactivity (Fig. 7*A*), but there appeared to be COMT-independent pathways as well, because tyrphostin AG1288 itself could increase such labeling (Fig. 7*B*), and AG1288 could at most only partially inhibit the volatile product formation stimulated by tyrphostin A25. We have not further characterized the liver enzymes involved in the COMT-independent pathway.

In Fig. 8, we present a model that can explain the results obtained in this work. In select tissues, COMT can methylate tyrphostin derivatives that then can be modified further into derivatives that release the methyl group as a volatile product upon base treatment and that can be covalently attached to proteins. The pathway shown in the model is not meant to be exclusive; other pathways employing similar chemistries are possible (see the figure legend and discussion below).

DISCUSSION

Tyrphostin Stimulation of Protein Methylation in Mammalian Cells—In the results presented above, we provide evidence that an apparent simple protein methylation event can originate from a more complex series of reactions. Although a methylation step is involved, it is not due to a protein methyltransferase but to COMT that methylates tyrphostin itself and the subsequent attachment of this species to proteins (Fig. 8). It is also possible that COMT can methylate tyrphostin-protein adducts directly. Additionally, what appears to be protein methyl ester formation is likely to reflect the base lability of methylated and oxidized tyrphostins (Fig. 8).

The reaction scheme shown in Fig. 8 explains why tyrphostins A25 and A51 are most active in protein labeling. These are the only tyrphostin derivatives tested that contain three adjacent hydroxyl groups (pyrogallol structures) and that participate in the mechanism shown in Fig. 8. The tyrphostins containing two adjacent hydroxyl groups cannot be oxidized to the *ortho*-quinone once methylated by COMT (28, 30, 31). Additionally, the pyrogallol derivatives may have a higher affinity for COMT (27). It is not clear, however, why some activity is seen

with tyrphostin A47, which does not have three hydroxyl groups.

Catechols are known to form protein adducts and to lead to protein cross-linking, presumably through *ortho*-quinone intermediates (32–34). Our observation of methylated species in the stacking gel in our SDS-PAGE analysis is consistent with tyrphostin-dependent protein cross-linking. That such cross-linking is largely specific to tyrphostins A25 and A51 is explained by the chemistry described in Fig. 8 where oxidation to the *ortho*-quinone can occur after the methylation reaction.

COMT and Tyrphostin Pharmacology—We have shown that a number of the commonly used tyrphostin protein-tyrosine kinase inhibitors undergo methylation by COMT. We have also shown that tyrphostin AG1288 is a potent COMT inhibitor, consistent with its nitrocatechol structure (27). These results suggest the possibility that COMT activity might attenuate or potentiate their kinase inhibitory action. Optimal kinase inhibition is generally seen with tyrphostins containing a 3,4-dihydroxylcatechol ring (35). For example, replacement of a hydroxyl group with a methoxy group on tyrphostin AG538 reduces its kinase inhibitor effect (36). Although catechol-containing tyrphostins are more active, they are generally less stable (see below) (36, 37).

Tyrphostins, including the species shown here to stimulate protein methylation, have been shown to have inhibitory effects on adenylate cyclase (16) and enzymes that use GTP as a cofactor (17). Tyrphostins, including A23 and A25, have also been seen to effect cellular GSH levels, reactive oxygen species production, and glutamate-stimulated cell death (38). It will be interesting to see if these effects may be linked to their stimulation of the reactions described in this study.

The inherent reactivity of the catechol moiety present in most active tyrphostin compounds can lead to instability. For example, tyrphostin A23 (Table 1) breaks down into two major oxidation products that are also protein-tyrosine kinase inhibitors (39). The more active product was found to be a dimer of A23 joined at the β-carbon, creating a di-catechol (39). This instability, which may include the chemistry described in this study, can limit the usefulness of the catechol-contain-

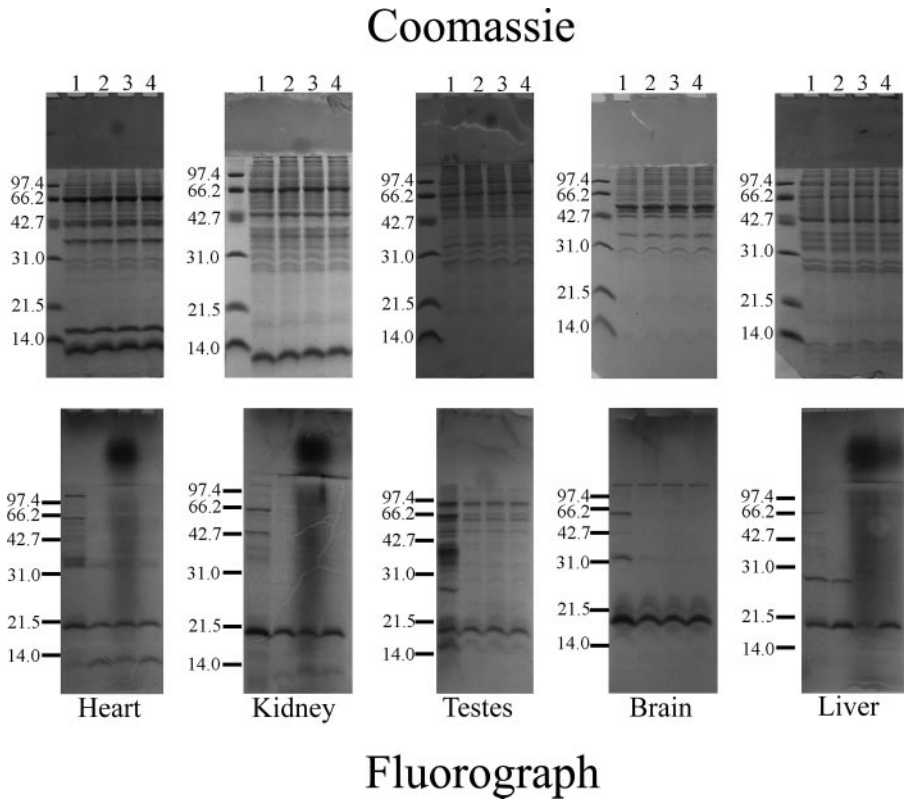


FIGURE 6. **SDS-PAGE analysis of the effect of the tyrphostin AG1288 COMT inhibitor on tyrphostin A25-stimulated protein labeling from [^{3}H]AdoMet in heart, kidney, testes, brain, and liver cytosol.** Mouse cytosolic extracts were incubated with [^{3}H]AdoMet and the polypeptides analyzed by SDS-PAGE as described in Fig. 2. Coomassie-stained gels are shown in the *upper panel*; a 2-month fluorograph is shown in the *lower panel*. Molecular weight markers, listed in Fig. 2, were electrophoresed in parallel lanes, and their positions are indicated on the *left* side of each gel panel. In each case *lane 1* is a control without further additions and *lane 2* is a control with the addition of 10% Me_2SO. In the samples electrophoresed in *lane 3* 100 μM tyrphostin A25 was added; in *lane 4* samples contained both 100 μM tyrphostin A25 and 100 μM of the COMT inhibitor tyrphostin AG1288.

TABLE 2

Endogenous COMT activity in mouse cytosolic extracts

Mouse tissue cytosol extracts were prepared and incubated with [^{14}C]AdoMet as described under "Experimental Procedures." The ability of tyrphostin A25 or catechol to serve as a methyl-accepting substrate was tested in the presence and absence of the COMT inhibitor tyrphostin AG1288. Me_2SO was added to control samples and inhibitor-less samples to maintain the levels in the tyrphostin A25/AG1288 samples. Radiolabeled catechol products were extracted using ethyl acetate and quantitated as described for the recombinant COMT assay under "Experimental Procedures." Average values ± S.D. are given for triplicate measurements.

Tissue	Methyl-accepting substrate					
	Control		Tyrphostin A25 (100 μM)		Catechol (1,2-benzenediol) (100 μM)	
	–	AG1288 (100 μM)	–	AG1288 (100 μM)	–	AG1288 (100 μM)
	pmol/min/mg protein					
Heart	1.8 ± 0.1	2.1 ± 0.3	4.7 ± 0.2	2.5 ± 0.3	7.0 ± 1.8	2.8 ± 0.3
Kidney	0.8 ± 0.0	0.9 ± 0.0	8.1 ± 0.6	1.6 ± 0.1	38.4 ± 1.3	1.5 ± 0.0
Testes	0.6 ± 0.0	0.7 ± 0.1	1.7 ± 0.1	0.8 ± 0.1	6.6 ± 0.3	0.8 ± 0.1
Brain	1.5 ± 0.0	1.7 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	2.6 ± 0.4	2.4 ± 0.3
Liver	1.3 ± 0.1	1.3 ± 0.1	19.4 ± 0.8	3.0 ± 0.3	55.2 ± 3.0	1.8 ± 0.1
No extract	2.0 ± 2.7	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1

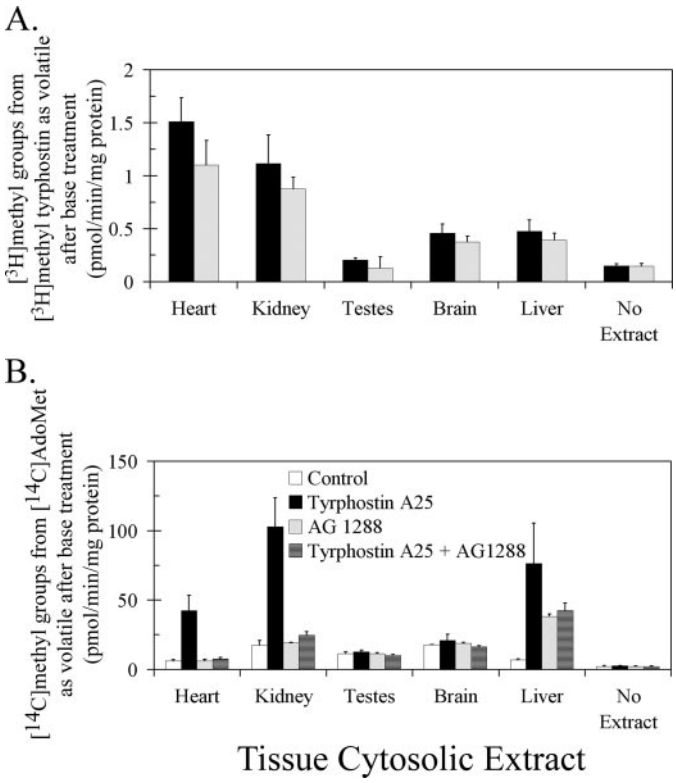


FIGURE 7. **Analysis by the vapor-diffusion assay of the effect of the tyrphostin AG1288 COMT inhibitor on tyrphostin-stimulated base-labile volatile radiolabeled methyl group formation in heart, kidney, testes, brain, and liver.** In *A*, tyrphostin A25 was prelabeled with ^{3}H-methyl groups in a 500-μl reaction mixture with recombinant COMT (0.001 units/μl) in buffer (10 mM Tris, pH 7.4, 1 mM $MgCl_2$, 500 μM dithiothreitol) in the presence of 2.5 μM [^{3}H]AdoMet (79 Ci/mmol) and 100 μM tyrphostin A25. The mixture was incubated at 37 °C for 60 min. An equal volume of water was added, followed by 1 ml of 2 N HCl. To extract the methylated tyrphostin A25, an equal volume of ethyl acetate was added and the sample spun at 14,000 × *g* for 5 min to separate the organic and aqueous layers. The top organic layer was collected, and the extraction was repeated three times, combining the organic layers. This material (^{3}H-methylated tyrphostin A25) was dried under vacuum, resuspended in Me_2SO to a concentration of 0.1 pmol of ^{3}H-methyl groups/μl, and then mixed with mouse tissue extracts for 37 °C for 60 min. Samples were incubated with the addition of either 10% Me_2SO (*black bars*) or 100 μM tyrphostin AG1288 (*gray bars*). In *B*, mouse tissue cytosol extracts were reacted with [^{14}C]AdoMet as described in Fig. 5 in the absence of tyrphostins (control, *white bars*), the presence of 100 μM tyrphostin A25 or AG1288 alone (*black* and *light gray bars*), or tyrphostin A25 and AG1288 together (*dark gray bars*). All samples were analyzed by the vapor-diffusion assay to determine base volatile radioactivity. In both panels, the average values of triplicate incubations are given with the standard deviation.

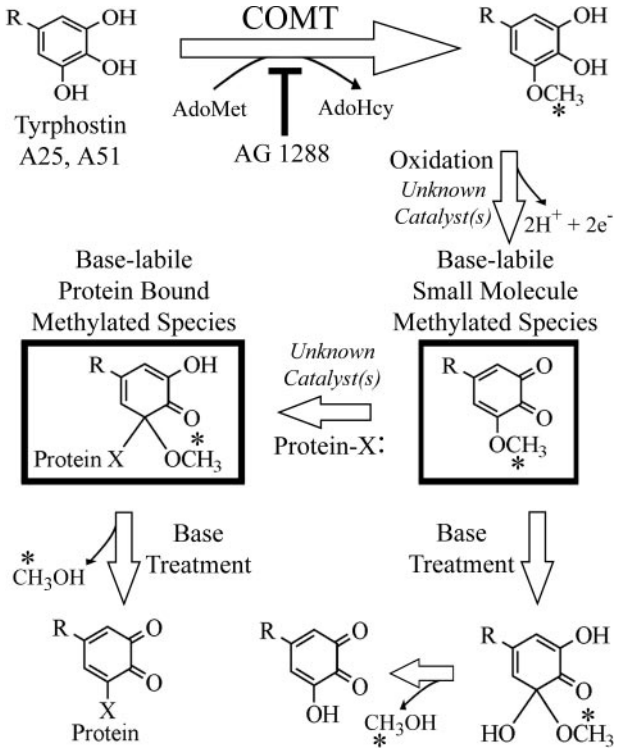


FIGURE 8. **A possible pathway for tyrphostin-stimulated protein labeling and base-labile volatile product formation.** In heart, kidney, testes, and liver, tyrphostin A25 and A51 are methylated by endogenous COMT activity from AdoMet. This enzymatic reaction is inhibited by tyrphostin AG1288. We propose that the methylated tyrphostin (methyl group identified by an *asterisk*) is oxidatively modified in heart and kidney extracts, either enzymatically (33, 45) or non-enzymatically (20, 46), to give an *ortho*-quinone derivative that can form adducts with proteins via Michael addition reactions (32). One example of such an adduct formation is shown; similar reactions giving other types of products are also possible. The projected products of these reactions, the small molecule methylated species and the protein-bound methylated species shown above, can be degraded in a base to labilize the methyl group as volatile methanol. Here, Michael addition of hydroxide ion to the small molecule-methylated species can yield an intermediate species that can form methanol and regenerate the *ortho*-quinone by a β-elimination mechanism (47). Similar chemistry can give methanol from the protein-bound methylated species. Other possible pathways can involve the initial formation of protein adducts with tyrphostins followed by methylation of the protein-tyrphostin complex with COMT. A distinct mechanism may allow for the small amount base-labile volatility seen with tyrphostin A47, where only two ring hydroxyl groups are present. These reactions shown here represent novel processes for the modification of proteins that mimic protein carboxyl methylation reactions catalyzed directly by protein-specific methyltransferases.

ing tyrphostins for the treatment of cancer and other proliferative diseases. In fact, the tyrosine kinase inhibitors that have progressed to clinical trials to date do not contain a catechol moiety (12, 14, 15).

Physiological Implications—Many cells contain endogenous catechols that can form covalent adducts with cellular proteins (19, 33, 34, 40–42). The results of this work suggest that COMT-modified catechols can also participate in these reactions. Both rat liver microsomes and tyrosinase catalyze the formation of covalent protein adducts with the catechols DOPA and dopamine (33). Interestingly, toxicity of the anticonvulsant drug phenytoin has been linked to its metabolism to a catechol and its subsequent oxidation to the *ortho*-quinone (43, 44). A number of "quinone-tanned" structures in nature, including eggshell matrices, biological cements, and mussel byssal threads, are also based on the oxidation of protein-bound catechols (45). In some of these systems, methylation by COMT can attenuate the oxidation and subsequent reactions (28, 30, 31); in other cases methylation may play a different role.

The results shown in this report suggest that care should be taken in analyzing protein methylation reactions in cellular extracts and intact cells radiolabeled with methionine or AdoMet. This is of particular concern, because the base lability of endogenous catechol methyl ether derivatives can mimic the properties of protein methyl esters. Furthermore, our results suggest that it may be important to consider the role of cellular methylation reactions in understanding the effects of catechol-containing tyrphostins used as experimental reagents and as possible therapeutics.

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