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-3H]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-3H]methionine or S-adenosyl-[methyl-14C]methionine stimulates the labeling of small molecules in addition to proteins. Base treatment of both protein and small molecule fractions releases volatile radioactive activity, 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.

We have been especially interested in identifying novel types of protein methylation reactions. In 1999, Bilodeau and Béliveau (10) demonstrated that S-adenosyl-[methyl-3H]methionine ([3H]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 3H-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 methylatation, 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...
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 Me2SO 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 10 mM. Methyl-α-DL-α-alcohol (pyrocatechol, 1,2-benzenediol) was obtained from Sigma-Aldrich and brought up in water to a stock concentration of 10 mM. Ammonium acetate, 1% bovine serum albumin, and 10% Triton X-100 were purchased from Sigma-Aldrich.

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 adjusted to pH 7.4, 1 mM MgCl2, 500 mM dithiothreitol with 1.25 μM [3H]AdoMet (Amersham Biosciences, 70 – 81 Ci/mmol, in Me2SO, 1 mM MgCl2, 500 μM dithiothreitol with 1.25 μM [3H]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 in a Beckman LS6500 scintillation counter.

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 [3H]AdoMet (11). To identify and characterize the methylated polypeptides, we first compared the fractionation of the 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. 1A and inset). Peaks corresponding to unreacted [3H]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-
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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 \(\mu\)mol \([^{3}H]\)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 \(\mu\)M 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 of the labeling solution.

A. Total \([^{3}H]\)methyl groups (pmol/fraction) as a function of elution time (min).

B. \([^{3}H]\)methyl groups as a function of elution time (min).

Analysis of the structures of the most active tyrphostin derivatives suggests the importance of three hydroxy groups on the aromatic ring present in A25 and A51. It is unclear, however, why A47 is more active than A25 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 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).

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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 derivatives 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 [14C]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 Me2SO 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.

<table>
<thead>
<tr>
<th>Tyrphostin</th>
<th>Structure</th>
<th>Vapor Diffusion Methylation Activity (pmol/min/mg protein)</th>
<th>COMT Activity (pmol/min/unit enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO control</td>
<td>-</td>
<td>8.6 ± 1.8</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>A25</td>
<td><img src="image" alt="Structure A25" /></td>
<td>44.9 ± 7.8</td>
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<td>A47</td>
<td><img src="image" alt="Structure A47" /></td>
<td>24.8 ± 6.5</td>
<td>2.45 ± 0.10</td>
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<tr>
<td>A51</td>
<td><img src="image" alt="Structure A51" /></td>
<td>99.1 ± 6.8</td>
<td>1.79 ± 0.18</td>
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<tr>
<td>A1</td>
<td><img src="image" alt="Structure A1" /></td>
<td>9.1 ± 0.7</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>A8</td>
<td><img src="image" alt="Structure A8" /></td>
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<td>0.05 ± 0.00</td>
</tr>
<tr>
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<td><img src="image" alt="Structure A9" /></td>
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<td>0.04 ± 0.01</td>
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<tr>
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<td>2.44 ± 0.01</td>
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<td>0.05 ± 0.01</td>
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<tr>
<td>B40</td>
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<td>2.98 ± 0.18</td>
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<tr>
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<td>3.06 ± 0.10</td>
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<td>3.11 ± 0.24</td>
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<td>3.06 ± 0.09</td>
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<tr>
<td>B56</td>
<td><img src="image" alt="Structure B56" /></td>
<td>11.5 ± 0.4</td>
<td>2.84 ± 0.29</td>
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COMT- and Tyrphostin-dependent Protein Methylation

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% Me2SO (white bars) or with buffer was used in place of cytosol in the presence of tyrphostin A25 (lined bars). Samples were analyzed directly by the vapor-diffusion assay (whole reaction) or after trichloroacetic acid (TCA) precipitation. Here, the reaction mixture was incubated with [3H]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.

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 [14C]AdoMet, tyrphostin, and cytosol by simply adding 3H-methylated tyrphostin A25 to the tissue extract. We thus

![Fluorograph](image1)

![Fluorograph](image2)
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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% Me2SO (white bars) using either no added methyl-accepting substrate (control, 10% Me2SO), 100 μM catechol (1,2-benzendiol), 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 14C-volatile product formation after base treatment of heart cytosol incubated with [14C]AdoMet. Mouse heart cytosol was incubated as described under "Experimental Procedures" in the presence of 100 μM tyrphostin AG1288 (black bars) or 10% Me2SO (white bars) with the addition of 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. 7A, significant stimulation of base-labile volatile radioactivity is seen in both heart and kidney cytosolic extracts with 3H-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. 7B the expected dependence upon COMT activity when [14C]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 3H-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 [3H]- or [14C]AdoMet-dependent methylation using SDS-PAGE analysis (Fig. 6) or the vapor-diffusion assay (Fig. 7B). However, when 3H-methylated tyrphostin A25 was used in a vapor-diffusion assay, activity was seen (Fig. 7A). Given the near absence of COMT activity in brain cytosolic extracts (Table 2), these results suggest that methylation in brain may be similar to both 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, 7A, and 7B), 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 adduction 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. 3H-Methylated tyrphostin A25 could stimulate a moderate production of base-labile volatile radioactivity (Fig. 7A), but there appeared to be COMT-independent pathways as well, because tyrphostin AG1288 itself could increase such labeling (Fig. 7B), 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 further modified into derivatives that release the methyl group as a volatile product under 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...
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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 Tyrophostin 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-dihydroxycatechol 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).

Tyrophostins, 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). Tyrophostins, 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-

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**Fluorograph**

**TABLE 2**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Methyl-accepting substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AG1288 (100 μM)</td>
<td>Tyrophostin A25 (100 μM)</td>
</tr>
<tr>
<td>Heart</td>
<td>1.8 ± 0.1</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.8 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>Testes</td>
<td>0.6 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>1.5 ± 0.0</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>No extract</td>
<td>2.0 ± 0.2</td>
<td>0.4 ± 0.0</td>
</tr>
</tbody>
</table>

**Coomassie**

**FIGURE 6.** SDS-PAGE analysis of the effect of the tyrphostin AG1288 COMT inhibitor on tyrphostin A25-stimulated protein labeling from [3H]AdoMet in heart, kidney, testes, brain, and liver cytosol. Mouse cytosolic extracts were incubated with [3H]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% Me2SO. 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.
This material (3H-methylated tyrphostin A25) was dried under vacuum, resuspended in Me2SO to a concentration of 0.1 pmol of 3H-methyl groups/μl, and then mixed with mouse tissue extracts for 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 x 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. Then, the extraction was repeated three times, combining the organic layers. The top organic layer was collected, and the extraction was repeated three times, combining the organic layers.

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 anticancer 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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REFERENCES