The short-lived enzyme S-adenosylmethionine decarboxylase uses a covalently bound pyruvoyl cofactor to catalyze the formation of decarboxylated S-adenosylmethionine, which then donates an aminopropyl group for polyamine biosynthesis. Here we demonstrate that S-adenosylmethionine decarboxylase is ubiquitinated and degraded by the 26 S proteasome in vivo, a process that is accelerated by inactivation of S-adenosylmethionine decarboxylase by substrate-mediated transamination of its pyruvoyl cofactor. Proteasome inhibition in COS-7 cells prevents the degradation of S-adenosylmethionine decarboxylase antigen; however, even brief inhibition of the 26 S proteasome caused substantial losses of S-adenosylmethionine decarboxylase activity despite accumulation of S-adenosylmethionine decarboxylase antigen. Levels of the enzyme’s substrate (S-adenosylmethionine) increased rapidly after 26 S proteasome inhibition, and this increase in substrate level is consistent with the observed loss of activity arising from an increased rate of inactivation by substrate-mediated transamination. Evidence is also presented that this substrate-mediated transamination accelerates normal degradation of S-adenosylmethionine decarboxylase, as the rate of degradation of the enzyme was increased in the presence of AbeAdo (5′-[(Z)-4-amino-2-butenyl]methylamino]-5′-deoxyadenosine) (a substrate analogue that transaminates the enzyme); conversely, when the intracellular substrate level was reduced by methionine deprivation, the rate of degradation of the enzyme was decreased. Ubiquitination of S-adenosylmethionine decarboxylase is demonstrated by isolation of His-tagged AdoMetDC (S-adenosylmethionine decarboxylase) from COS-7 cells co-transfected with hemagglutinin-tagged ubiquitin and showing bands that were immunoreactive to both anti-AdoMetDC antibody and anti-hemagglutinin antibody. This is the first study to demonstrate that AdoMetDC is ubiquitinated and degraded by the 26 S proteasome, and substrate-mediated acceleration of degradation is a unique finding.

S-Adenosylmethionine decarboxylase (AdoMetDC) is a key enzyme in the biosynthesis of the ubiquitous polyamines spermine and spermidine, organic cations that are absolutely required for normal cell proliferation and differentiation (1, 2). AdoMetDC catalyzes decarboxylation of S-adenosylmethionine (AdoMet), which then provides aminopropyl groups for either spermidine and spermine synthesis (3). Spermidine synthase joins an aminopropyl group derived from decarboxylated AdoMet (dcAdoMet) with putrescine to produce spermidine. Similarly, an aminopropyl group from dcAdoMet is attached to spermidine by spermine synthase to generate spermine.

Mammalian AdoMetDC is synthesized as a proenzyme (38 kDa) that autokatalytically cleaves to form the α (30.7 kDa) and β (7.7 kDa) subunits in a reaction that simultaneously forms the pyruvoyl cofactor from an existing internal serine residue at the amino terminus of the newly cleaved α subunit (4). The mature catalytically active enzyme consists of a dimer (α₂β₂) of these subunits (5). Cellular AdoMetDC activity is tightly regulated at multiple levels: transcription of the AdoMetDC gene is repressed by spermidine, the translation of its mRNA is negatively regulated by spermine (6), and the processing of the proenzyme and the catalytic activity of the final enzyme are stimulated by the spermidine precursor putrescine (7, 8). It has been reported that the half-life of AdoMetDC is inversely related to the cellular content of spermidine and spermine (9–11), so that when the levels of polyamines are high, increased degradation of AdoMetDC serves as another important control mechanism in maintaining AdoMetDC activity and therefore polyamine levels (3, 5). The half-life of AdoMetDC is usually 1–3 h in most cell lines and tissues (13–18), although an AdoMetDC half-life of only 3 min has recently been reported for the parasite Crithidia fasciculata (19). In vivo the rapid degradation of AdoMetDC is inhibited by the binding of activity inhibitors such as methylglyoxal bis(guanylyl)hydrazone, aminoguanidine, S-methyl-5′-methylthioadenosine, or 4-amidinodan-1-one 2′-amidino hydrazone (5, 14, 20, 21). The mechanisms for changes in AdoMetDC degradation rate in response to inhibitors or other stimuli are not understood. In addition to loss of AdoMetDC activity through degradation of the enzyme (loss of antigenic AdoMetDC), it is also known that AdoMetDC activity can be lost by permanent covalent inactivation of the enzyme in a process known as substrate-mediated transamination. Transamination transfers the amine group from the substrate AdoMet, converting the covalently linked pyruvoyl cofactor to an alanine (22, 23). The depletion of intracellular AdoMet by the presence of the AdoMet synthase inhibitor L-2-amino-4-methoxy-cis-but-3-enoic acid has been reported to lengthen the half-life of AdoMetDC activity loss 3-fold (15), suggesting that the rate of this inactivation might be related to the intracellular concentrations of the substrate AdoMet, although...
though direct stabilization effects of the L-cisAMB could not be completely ruled out in those experiments. The fact that the measured rate of AdoMetDC activity loss is often faster than the rate of antigen loss also suggests that it could be this inactive form of AdoMetDC that is recognized by intracellular degradation machinery, or at least that this inactive form may be preferentially degraded. There are multiple systems that can mediate the degradation of intracellular proteins; however, the ATP-dependent ubiquitin 26 S proteasome pathway is thought to account for the degradation of the great bulk of short-lived or abnormally folded intracellular proteins, as well as of long-lived proteins (24, 25).

In this report, we investigate the proteolytic mechanisms responsible for the degradation of AdoMetDC in COS-7 cells in vivo and the effects of substrate levels and transamination on that degradation. Our results indicate that AdoMetDC is ubiquitinated and degraded by the 26 S proteasome. Although antigenic protein was clearly stabilized following inhibition of the 26 S proteasome or following ATP depletion, the rapid disappearance of catalytic activity following cycloheximide administration was not inhibited by either of these treatments. In addition, we have obtained evidence that the inactive (transamminated) form of the AdoMetDC enzyme is more rapidly degraded than the active form.

EXPERIMENTAL PROCEDURES

Materials—Enhanced chemifluorescent (ECF) detection reagent for Western blotting was purchased from Amersham Biosciences. MG-132, lactacystin, calo-lactacystin β-lactone, and calpain inhibitor II ALLM were from Calbiochem. Dulbecco’s modified Eagle’s medium (DMEM) and an MEM Select-Amine kit were purchased from Invitrogen. Fetal Calf Serum (FCS) was from HyClone (Logan, UT). Polyvinylidene difluoride (PVDF) membrane was obtained from Osmonics (Westborough, MA). DNA isolation kits and NTA magnetic agarose beads (5% slurry in lysis buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The next morning complexes were precipitated by centrifugation and washed three times with washing buffer (50 mM M glycerol, 250 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). To check for contamination, DNAs were digested with BamHI as described by Mann et al. (27). Brieﬂy, the lysates were cleared by debris by centrifugation at 11,000 rpm for 4°C for 10 min. After incubation with preimmune serum for 1 h (preclearing step) and then protein-A (30-min incubation), the lysates were incubated with anti-AdoMetDC polyclonal antibody for 3 h at 4°C and then protein A for 1 h. The complexes were precipitated by centrifugation and washed three times with washing buffer 1 (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate) and once with washing buffer 2 (10 mM Tris-HCl, pH 7.5). The immunoprecipitated complexes were then separated by SDS-PAGE on a 12.5% gel. After electrophoresis, the gel was washed extensively with fixing solution (30% methanol, 7% glacial acetic acid, 10% glycerol) for 3 h, dried, and exposed for 1 week. Film (Hyperfilm H, Amersham Biosciences) was used for autoradiography. Enhanced chemiluminescent (ECL) detection reagent for Western blots was obtained from Amersham Biosciences. All other chemicals were obtained from Fisher.

Plasmids Used—The His-tagged human AdoMetDC cDNA portion of the previously produced bacterial expression construct pQE-HISSAMI (22) was subcloned into the mammalian expression vector pCDNA3.1/Zeo (-) (Invitrogen) using PstI and XhoI restriction enzyme HI. DNA from COS-7 cells was grown in 10-cm dishes at a density of ~8 × 10⁵ cells. After several days’ growth, cells were transfected using LipofectAMINE reagent according to the manufacturer’s protocol (Invitrogen). To investigate whether ubiquitin was conjugated to His-AdoMetDC, cells were transfected with 4.1 µg of the HA-ubiquitin-expressing plasmid MT123 plus 1.4 µg of His-AdoMetDC-expressing plasmid. Control cells were transfected with 4.1 µg of His-AdoMetDC plasmid and 1.4 µg of empty pcDNA3.1/Zeo (-) vector. After 48 h of transfection, cells were treated with 50 µg MG-132 to inhibit the 26 S proteasome or vehicle control (0.1% MeSO) for 4 h. Cells were harvested in imidazole harvesting buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 5 mM N-ethylmaleimide, pH 8.0) and lysed by three quick freeze-thaw cycles in liquid N₂. His-AdoMetDC was then bound to Ni-NTA magnetic agarose beads according to the manufacturer’s instructions (Qiagen). After binding, the beads were washed three times with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). His-AdoMetDC was then eluted from the beads by elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) followed by protein A precipitation and Western blotting. Transfected human AdoMetDC or His-tagged human AdoMetDC had degradation rates in COS-7 cells 2 days after transfection that were almost identical to the degradation rate of endogenous COS-7 cell AdoMetDC (results not shown).

Western Blotting—Amersham Biosciences’ ECF detection system was used to visualize the Western blotting protein bands. Protein bands were separated by electrophoresis on 12.5% polyacrylamide gels under denaturing conditions in 0.1% SDS (28). The proteins were then transferred to PVDF membranes at 30 V overnight at 4°C in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The next morning, the voltage was raised to 90 V for an additional hour to complete the transfer. The blots were then incubated with rabbit polyclonal antibody to HA-AdoMetDC antiserum (1:5000 dilution) overnight in TBS-T (Tris-buffered saline-Tween 20). The blots were then extensively washed with TBS-T and incubated with anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:10,000 dilution) for 1 h. To detect HA-ubiquitin-
AdoMetDC conjugates or HA-ubiquitin expression, blots were probed with a monoclonal anti-HA monoclonal antibody (1:500 dilution) for 1 h. The blots were extensively washed with TBS-T and then incubated with anti-mouse alkaline phosphatase-conjugated secondary antibody (1:500 dilution) for 1 h. Finally, the blots were developed with ECF substrate for 3–5 min. The fluorescence developed was scanned with an Amersham Bioscience FluorImager using a 570-nm filter, and specific bands were quantitated using ImageQuant software.

**AdoMetDC and ODC Activity Assays**—Cells were harvested in AdoMetDC harvest buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 2.5 mM putrescine, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) and lyzed by three quick freeze-thaw cycles using liquid N$_2$. Cell lysates were centrifuged at 11,000 rpm for 10 min, and after removing a supernatant for protein determination, the remainder of the cleared supernatants were preserved at −80 °C until assay. Equal amounts of protein from supernatants were assayed for AdoMetDC activity by measuring the amount of $[^{14}C]$AdoMet converted into $^{14}$CO$_2$ over 1 h of shaking at 37 °C. Activities of AdoMetDC were determined using the amount of $[^{14}COOH]$AdoMet converted into $^{14}$CO$_2$ over 1 h of shaking at 37 °C. The assay mix consisted of 1.25 mM DTT, 50 mM sodium phosphate buffer, pH 6.8, 2.0 mM putrescine, and 3.84 µCi $[^{14}COOH]$AdoMet (57 mCi/mmol) in a total volume of 0.25 ml. $^{14}$CO$_2$ was trapped in 0.3 ml of hyamine hydroxide contained in a center well (Kontes) located above the reaction mix in the middle of sealed assay tubes; after 1 h of incubation, 0.5 ml of 5N H$_2$SO$_4$ was injected through the rubber seal to stop the reaction. After a further 20 min of shaking at 37 °C, the hyamine hydroxide-containing center well was placed into 10 ml of scintillation mixture and counted. The specific activity was calculated as the amount of substrate converted (pmol) per mg of cell extract protein in 1 h at 37 °C (29). ODC activity was similarly measured as the amount of [1-14C]ornithine converted into $^{14}$CO$_2$ over a 1-h incubation at 37 °C. The ODC assay mix contained 50 mM pyridoxal phosphate, 62.5 mM Tris-HCl, pH 7.5, 3.125 mM DTT, and 0.1 µCi of [1-$^{14}$C]ornithine (40–60 µCi/mmol) in a total volume of 0.2 ml (30).

**AdoMet Analysis**—Cells were harvested in AdoMetDC harvest buffer and lyzed by three quick freeze-thaw cycles in liquid N$_2$. After being centrifuged at 11,000 rpm for 10 min, the supernatant was transferred to a new tube and saved at −80 °C until HPLC analysis. Aliquots containing equal amounts of protein were adjusted to 0.2 N perchloric acid and centrifuged again at 11,000 rpm for 10 min to remove acid-insoluble proteins. The supernatant was then used for the determination of AdoMet concentration. The HPLC system consisted of a Waters 600E multisolute delivery system equipped with a Waters 484 tunable absorbance detector and a 25-cm × 2.1-mm column (packed with PO-RS R2 medium (10-µm diameter; Perseptive Biosystems)). A gradient system run at 1.3 ml/min was used to separate AdoMet from other UV-absorbing compounds. Mobile phase A (Solution A) consisted of 90 mM α-phosphoric acid and 10 mM SDS. Mobile phase B (Solution B) consisted of 30% acetonitrile and 70% 0.2 mM sodium acetate, pH 4.5, 10 mM SDS solution. The column was equilibrated with 20% Solution B and 80% Solution A for 4 min and then sample was injected followed by a linear gradient change of solvent composition to 50% Solution B and 50% Solution A over 2 min, to 60% Solution B and 40% Solution A over 2 min, to 75% Solution B and 25% Solution A over 3 min, to 100% Solution B over 7 min, and finally washed isocratically at 100% Solution A for 15 min at a flow rate during the wash only of 2.0 ml/min. AdoMet and other peaks were detected at 260 nm. A standard curve was obtained as a function of known concentration of AdoMet and the corresponding peak area using linear regression with Prism 3.0 software, and unknown AdoMet concentrations were calculated from their peak areas using the standard curve.

**Protein Determination**—The protein concentration was determined using the Bio-Rad dye binding microassay. Bovine serum albumin at 1–200 µg/ml was used as standard (31).

**Half-life Calculation**—The half-life of AdoMetDC or ODC was determined by stopping de novo protein synthesis by cycloheximide (0.4 mM) or by following the disappearance of radioactivity incorporated into the protein for appropriate times after radioactive precursor amino acids were removed. The log values of the specific activities or of the amount of AdoMetDC antigen expressed as the percentage of amount at time 0) was plotted against time. With Prism 3.0 software (GraphPad, San Diego, CA) using Student’s two-tailed $t$ test. Results were considered significant at $p < 0.05$. For determination of significance of differences in half-lives, the calculated degradation rate constants and their associated standard errors were used to calculate $t$ values. When multiple conditions were compared, a one-way ANOVA was performed, and if significant overall differences in sample means were found ($F$ test value $p < 0.05$), the Bonferroni post-test was used to determine the significance of individual treatment differences from controls.

**RESULTS**

We first examined the effects of depletion of intracellular ATP on the loss of AdoMetDC antigen and activity. COS-7 cells were depleted of ATP (to 8–15% of control levels, as determined by the firefly luciferase method) with glucose-free medium, NaCN, and 2-deoxyglucose as described under “Experimental Procedures.” After 1 h in NaCN and 2-deoxyglucose, de novo protein synthesis was stopped by the addition of cycloheximide to the cells, and remaining antigenic AdoMetDC and AdoMetDC enzymatic activity were measured at the time points after cycloheximide addition indicated in Fig. 1. As seen in panel A of Fig. 1, ATP depletion completely inhibited degradation of AdoMetDC antigen; however, as seen in panel B, ATP depletion had no significant stabilizing effect on AdoMetDC activity, which disappeared at the same rate in the presence of normal or reduced ATP.
We then examined the effect of several proteasome inhibitors on AdoMetDC degradation. Exponentially growing COS-7 cells were treated at time 0 with the peptidyl aldehyde protease inhibitor MG-132 and cycloheximide, and disappearance of antigenic protein was then followed for 3 h. Control cells were treated with cycloheximide and Me$_2$SO (vehicle control for the proteasome inhibitor), which did not change the half-life compared with cells treated with cycloheximide alone (results not shown). From each time point, equal amounts of protein were separated by SDS-PAGE followed by Western blot analysis (Fig. 2A). As plotted in Fig. 2B, a half-life of 1.9 h for AdoMetDC antigen disappearance was observed in control cells, whereas the enzyme was completely stable when the 26 S proteasome was inhibited by MG-132. The enzyme was also stabilized when the cells were treated with the highly specific proteasome inhibitor lactacystin or its active metabolite clasto-lactacystin β-lactone (data not shown). In contrast, the results from additional experiments (Fig. 2C) demonstrate that treatment of cells with the calpain inhibitor II (ALLM) or the metalloprotease inhibitor (1,10-phenanthroline) did not inhibit the degradation of AdoMetDC, whereas MG-132 completely prevented degradation (the experiments shown in Fig. 2C measured disappearance of radiolabeled rather than Western-blotted AdoMetDC, but no differences in the measured degradation rates were observed between the two methods). Similarly, pre-treatment of cells with the lysosomal protease inhibitor NH$_4$Cl showed no effects on AdoMetDC degradation (results not shown). Thus, neither calpains nor metalloproteases nor lysosomal proteases appear to have a significant role in normal intracellular AdoMetDC degradation. Taken together, these results indicate that the 26 S proteasome is responsible for the normal turnover of AdoMetDC in COS-7 cells.

We also investigated the loss of endogenous AdoMetDC catalytic activity following the treatment of cells with the proteasome inhibitors. The disappearance of AdoMetDC activity was approximately twice as rapid as that of AdoMetDC antigen, and as can be seen in Fig. 3A, treatment of cells with MG-132 did not prevent the loss of AdoMetDC activity after protein synthesis was inhibited, even though MG-132 clearly stabilized the antigenic protein in the same cell lysates (Fig. 2). This suggests that essentially none of the disappearance of AdoMetDC activity under these conditions is because of loss of AdoMetDC antigen. As a positive control for the expected stabilization of activity of 26 S proteasome substrates when degradation is inhibited, the half-life of ODC, another 26 S proteasome substrate, was measured from the same cell extracts. ODC activity (as well as antigen) was strongly stabilized by 26 S proteasome inhibition (activity half-life increased 4.4-fold; see Fig. 3B).

Both the activities and antigenic amounts of multiple short-lived ubiquitin-proteasome pathway substrates (as well as ubiquitin-modified forms) are observed to increase rapidly in the presence of 26 S proteasome inhibitors when normal protein synthesis is allowed to continue (no cycloheximide included to inhibit protein synthesis) (for example see Refs. 32–35). However, not only did the presence of proteasome inhibitors fail to inhibit AdoMetDC activity loss in the presence of cycloheximide (whereas AdoMetDC antigen was clearly stabilized), but inhibition of the 26 S proteasome in the absence of cycloheximide actually led to significant losses of AdoMetDC activity. This can be seen as in Fig. 4, where AdoMetDC activity was 2.7-fold lower in COS-7 cells treated with MG-132 alone compared with the control cells. The same result was consistently obtained in all cell types that were used in this study. AdoMetDC activity was also 2-fold lower when COS-7 cells were treated with lactacystin, indicating that the inactivation is specifically because of inhibition of the 26 S proteasome. In contrast, ODC activity was increased 2.5-fold in the same 26 S proteasome-inhibited lysates (data not shown). Neither MG-132 nor lactacystin inhibited the activity of in vitro synthesized human AdoMetDC with up to 3 h of preincubation, demonstrating that inhibition of the 26 S proteasome activity rather than direct effects of the inhibitors themselves caused the observed inactivation of AdoMetDC. The increased antigen amounts ob-
two to four separate plates; where error bars are not visible they are smaller than the size of the symbol. Harvested and lysed in AdoMetDC harvest buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 2.5 mM putrescine, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) by three freeze-thaw cycles. Panel A, AdoMetDC activity was assayed in the supernatants as release of radiolabeled $^{14}$CO$_2$ from $S$-adenosyl(carboxyl-$^{14}$C)methionine. Each data symbol is the mean ± S.E. from three to four separate plates; where error bars are not visible they are smaller than the size of the symbol. Panel B, ODC activity was assayed by measuring the release of $^{14}$CO$_2$ from [1-$^{14}$C]ornithine. Each data symbol is the mean ± S.E. from two to four separate plates; where error bars are not visible they are smaller than the size of the symbol. Open bars, control cells; black bars, MG132-treated.

**Fig. 3. Effect of proteasome inhibition on AdoMetDC and ODC activity half lives.** Cycloheximide (0.4 mM) or 0.1% Me$_2$SO (control) for 4 h. Samples were processed as described for Fig. 2A. ODC activity was decreased by 0.2–0.6-fold compared to the control, while AdoMetDC activity was decreased by 0.4–0.6-fold compared to the control. Each data symbol represents the mean ± S.E. from two to four separate plates; where error bars are not visible they are smaller than the size of the symbol. Black bars, control cells; gray bars, MG132-treated.

**Fig. 4. Effect of inhibition of the 26 S proteasome on AdoMetDC catalytic activity in COS-7, T84, and H4IIE cells.** Cells were treated with MG-132 (50 μM) or 0.1% Me$_2$SO (control) for 4 h and lysed in AdoMetDC harvest buffer by three quick freeze-thaw cycles, followed by centrifugation (11,000 rpm for 10 min) to remove cell debris. AdoMetDC activity was measured in the supernatant as release of radiolabeled $^{14}$CO$_2$ from $S$-adenosyl(carboxyl-$^{14}$C)methionine, as described in detail under “Experimental Procedures.” Open bars, COS-7 cells; black bars, T84 cells; gray bars, H4IIE cells. Each bar shows the mean ± S.E. from four independent determinations; each of the MG132 means is significantly different from their corresponding controls (p < 0.01, Student’s t test).

served under these conditions must therefore represent the accumulation of inactive AdoMetDC protein. This in turn suggested that inhibition of the 26 S proteasome is accelerating some intracellular reaction that inactivates AdoMetDC.

With the exception of substrate AdoMet-mediated transamination and related alkylations (22, 23, 36) and a suggestion of a modified residue in bovine AdoMetDC (37), no other posttranslational modifications of mature (cleaved) AdoMetDC have been described. Transamination by either the substrate AdoMet or the catalytic product dcAdoMet converts the covalently linked pyruvoyl cofactor to alanine and permanently inactivates AdoMetDC (22, 23), which has led to the suggestion that the rapid turnover of AdoMetDC may be teleologically explained by the need to remove this constantly forming pool of inactive enzyme (5). The intracellular concentration of AdoMet is below the Km for the AdoMetDC enzyme, so an increase in the substrate concentration would increase the number of catalytic turnovers proportionally. Because it appears that substrate-mediated transamination occurs as a relatively constant percentage of catalytic turnovers (22, 23), we reasoned that an increase in AdoMet level could also increase the rate of enzyme inactivation. We therefore measured AdoMet levels following inhibition of the 26 S proteasome by MG-132 (50 μM for 4 h). It was found that significant increases in AdoMet levels occurred even in this short time period; as seen in Fig. 5A, the AdoMet level increased 2- and 6-fold in COS-7 and T84 cells, respectively.

A similar increase (2.2-fold) was observed in COS-7 cells after 2 h of cycloheximide treatment (0.4 mM; see Fig. 5A). This latter result is as expected, because methionine serves as the biological precursor for AdoMet synthesis, and this pathway competes for methionine with protein synthesis (38); when the major pathway of methionine incorporation into protein is inhibited by cycloheximide, the pool of methionine available for AdoMet synthesis.
would increase. The opposite effect would be expected from inhibition of protein degradation, however, because this would tend to prevent recycling of methionine in protein back to free methionine available for AdoMet synthesis; nonetheless, inhibition of 26 S proteasome degradation by MG-132 clearly increased intracellular AdoMet levels in multiple experiments. This observed increase in AdoMet level is consistent with an accelerated rate of AdoMet-mediated inactivation of AdoMetDC activity, which may account for the lowered AdoMetDC activity observed despite AdoMetDC antigen accumulation.

To more directly test whether transamination plays a role in AdoMetDC antigen degradation, as well as the known effect of enzyme inactivation, AdoMetDC activity in COS-7 cells was completely inactivated by pretreatment for 1 h with 25 μM AbeAdo, a treatment that we have shown previously inactivates AdoMetDC through transamination of the pyruvoyl cofactor (39). AdoMetDC antigen disappearance after cycloheximide addition was then followed by Western blot analysis. The half-life of this transaminated AdoMetDC was found to differ significantly from that in controls (Fig. 6, A and B, 1.5 h in AbeAdo-pretreated cells as compared with 2.7 h in the control cells, p = 0.03). This is consistent with transamination triggering a more rapid degradation of AdoMetDC.

Although various attempts to raise intracellular AdoMet levels were unsuccessful, we had observed that AdoMet level could be reduced by about 80% in COS-7 cells by 1 h of methionine deprivation, whereas leucine deprivation did not affect the AdoMet level. Therefore, next we investigated whether AdoMetDC half-life was lengthened when AdoMet levels were lowered by brief methionine deprivation. COS-7 cells were deprived of methionine for 1 h before labeling with [35S]Met/Cys. Control cells were deprived of leucine for 1 h and labeled with [3H-4,5]Leu. Cells were harvested at indicated time points after removal of radiolabeled amino acid, and the remaining radiolabeled AdoMetDC 31-kDa band can be seen in Fig. 6C. With the remaining band densities from duplicate experiments plotted in Fig. 6D, AdoMetDC half-life in leucine-deprived cells was observed to be much shorter (1.3 h) than the half-life in methionine-deprived cells (3.1 h). Thus, decreasing the amounts of the methionine precursor and substrate AdoMet through simple brief manipulation of medium components led to a significant inhibition of AdoMetDC degradation rate (p = 0.0046).

Proteasome-mediated degradation of cellular proteins was originally thought to require tagging of target proteins by a polyubiquitin chain (24). The first exception discovered to this requirement was the 26 S proteasome-mediated but ubiquitin-independent degradation of ODC (40), whose levels and degradation rate generally rise and fall with those of AdoMetDC. Therefore, it was of clear interest to examine whether AdoMetDC is ubiquitinated in vivo and whether the antizyme that mediates the ubiquitin-independent degradation of ODC (40) might also bind to AdoMetDC. In a series of co-immunoprecipitation experiments in which in vitro-produced Antizyme was incubated with in vitro-produced ODC and/or AdoMetDC, anti-Antizyme antibodies quantitatively co-precipitated ODC and vice versa; however, no co-precipitation of AdoMetDC was observed with anti-Antizyme antibodies nor was Antizyme co-precipitated with anti-AdoMetDC antibodies. This suggests that no strong antizyme-AdoMetDC interactions occur, although an interaction requiring additional intracellular components not found in the rabbit reticulocyte lysates used for the in vitro production of the proteins would have been missed in those analyses.

8 W. A. Martin and B. A. Stanley, unpublished results.

In contrast, in initial experiments where COS-7 cell lysates were immunoprecipitated with anti-AdoMetDC antiserum followed by detection with anti-ubiquitin antibody, or in the reciprocal experiment, common cross-reactive bands were detected; however, because the general background was high using the anti-ubiquitin antibodies in those experiments, we decided to use an additional approach to verify these results. HA-tagged ubiquitin has been shown previously (26) to function like untagged ubiquitin in vivo, and His-tagged human AdoMetDC is degraded in transfected COS-7 cells at a very similar rate to that of the endogenous enzyme (results not shown). In the following experiments, COS-7 cells were cotransfected with HA-ubiquitin + His-AdoMetDC or as controls
Although both AdoMetDC and ODC activities are similarly regulated during cell proliferation, they are clearly targeted to the 26 S proteasome by different mechanisms. The evidence presented in the current work shows that AdoMetDC is degraded by the 26 S proteasome and that it has no obvious interaction with antizyme based on co-precipitation experiments. AdoMetDC is certainly ubiquitinated, and high molecular mass poly-ubiquitin tagged forms of AdoMetDC accumulate when the 26 S proteasome is inhibited (for example see lane 5 versus lane 4 in Fig. 7A). A recent report (42) of identical rates of c-Fos degradation with or without accompanying ubiquitination casts doubt on the idea that demonstrating poly-ubiquitinated forms of a 26 S proteasome-degraded protein is equivalent to demonstrating ubiquitin dependence of such degradation; however, the simplest explanation of the observed current results is that the 26 S proteasomal degradation of AdoMetDC is ubiquitin-dependent. Further tests will be necessary to see whether AdoMetDC degradation rates are altered in ubiquitination temperature-sensitive mutants such as those used in the c-Fos experiments.

In the presence of HA-ubiquitin, the amount of His-AdoMetDC protein was higher compared with the amount of His-AdoMetDC expressed alone. Several experiments were carried out to eliminate any potential contribution from different plasmid quality or amount of plasmid transfected; however, the same result (increased AdoMetDC amount when co-transfected with HA-ubiquitin) was consistently obtained. The presence of more ubiquitin because of HA-ubiquitin transfection was also shown to increase the amount of polyubiquitinated material, consistent with the idea that ubiquitin in COS-7 cells may be limiting for general poly-ubiquitination or other ubiquitin-mediated processes. Also consistent with the idea that free ubiquitin may in some cases be limiting is the report that very little free ubiquitin exists in other cell types; instead ubiquitin exists almost completely as thiol esters attached to components of the ubiquitin conjugation pathway used by the cell for targeting proteins for degradation (43). By increasing the available free ubiquitin, transfection of HA-Ub in the current experiments could also affect the levels of ubiquitin tagging of other cellular components not being targeted for degradation. One such potential target of this hypothesized increased ubiquitination upon HA-Ub transfection would be the large ribosomal subunit L28, whose increased ubiquitination is at least correlated with increased rates of protein synthesis. L28 has been observed to be strongly ubiquitinated in S phase (44), when protein synthesis rates are higher (45), and to be ubiquitinated to a much lower extent in G1 phase, when protein synthesis rates are lower. This effect of higher levels of ubiquitination of L28 (or some other component) might potentially act on the translational efficiency of particular mRNAs as well, and AdoMetDC activity has also been found to increase in S phase (where L28 ubiquitination is more strongly ubiquitinated) in Chinese hamster ovary cells without a corresponding increase in mRNA levels (46), consistent with higher translational efficiency and/or decreased degradation rate. The mechanism leading to the observed higher accumulation of AdoMetDC with higher Ub levels in COS-7 cells remains to be determined, but the effect does not appear to be because of some simple artifact such as changes in transfection efficiency, because the quality and quantity of plasmid DNA transfected was rigorously controlled, and the effect was seen in repeated experiments.

Although AdoMetDC antigen loss is completely prevented by 26 S proteasome inhibition, AdoMetDC activity continued to disappear at the same rate as in control cells. This observation of loss of AdoMetDC activity occurring at the same rate
whether antigen degradation is prevented (in which case activity loss arises from inactivation only) or not (activity loss arises from both inactivation plus degradative losses of active enzyme) indicates that inactive AdoMetDC must be preferentially degraded; if this were not the case, then the rate of loss of activity would have to be greater when degradation of antigen,
and therefore active enzyme is included as a source of loss of activity. An accelerated loss of AdoMetDC activity following 26 S proteasome inhibition is also correlated with a significant increase in intracellular levels of the substrate AdoMet. Although inactivation because of an initial small (non-26 S proteasome) proteolytic clip cannot be completely ruled out by the current results, the accumulated evidence from our work and that from other laboratories (22, 23, 36) points to a substrate-mediated transamination of the covalently linked pyruvyl cofactor (and possibly additional accompanying alkylation events) as the cause of the observed inactivation. Taken together, these results strongly suggest that the enzyme may be transaminated before complete degradation by the 26 S proteasome and that such transamination may serve as a trigger for accelerated degradation.

The hypothesis that higher substrate AdoMet levels may increase transamination of the enzyme, in turn leading to more rapid degradation of AdoMetDC, is supported by the results of two other experiments. First, when the intracellular AdoMet level was reduced by methionine deprivation, the AdoMetDC half-life was much longer than when the cells were deprived of leucine (which does not affect the AdoMet level). A similar result was reported previously in Walker carcinoma and TLX5 lymphoma cells, where methionine deprivation (but not serine or glycine deprivation) caused an increase in AdoMetDC activity because of stabilization of the enzyme, although in that case the authors concluded that this effect was not because of (unmeasured) changes in AdoMet concentration, because the AdoMet synthase inhibitor cycloleucine did not stabilize AdoMetDC (47). In contrast, Krämer et al. (15) indicated that AdoMetDC activity was greatly stabilized (half-life of 310 min compared with 65 min in untreated controls) after treatment of L1210 cells with the AdoMet synthase inhibitor 1,2-amino-4-methoxy-cis-but-3-enoic acid (which resulted in 95% depletion of AdoMet in the presence of decreased methionine concentration in the medium), although direct effects of the inhibitor on AdoMetDC half-life could not be completely ruled out.

Second, direct evidence of an effect of transamination was shown by demonstrating that the AdoMetDC antigenic protein half-life was shorter in the presence of the transaminating AdoMetDC inhibitor AbeAdo, a result in direct contrast to the stabilizing effects of other non-transaminating AdoMetDC inhibitors (11, 48–50). Particularly striking is the contrast between the destabilizing effects of the transaminating AbeAdo shown here and the stabilizing effects of the inhibitor 5′-deoxy-5′-[N-methyl-N-(3-hydrizinopropyl)]laminoadenosine (48), which we have previously shown (39) also covalently modifies the pyruvate cofactor with the addition of a hydrazone moiety. Because many of the other stabilizing inhibitors act by non-covalently blocking substrate access to the catalytic site of the enzyme and therefore preventing AdoMetDC activity (51), transamination would also be prevented, thus accounting for the AdoMetDC antigen-stabilizing effects of these other inhibitors, whereas the transamination by AbeAdo causes destabilization.

These results also call into question whether the true intracellular half-life of AdoMetDC antigen or activity is being measured by classic half-life determination experiments, in which either antigen disappearance after cycloheximide administration is followed (Western blot approach) or in which radiolabeled protein disappearance is followed after 35S-methionine labeling in pulse-chase experiments. In the former approach, the increased AdoMet levels observed in several cell types after administration of cycloheximide would lead to more rapid transamination of AdoMetDC than would be occurring at normal intracellular AdoMet levels and therefore show a more rapid degradation rate than the normal in vivo rate; similarly, we have repeatedly observed that the flooding dose of unlabeled methionine following a 35S-methionine labeling period also causes a significant increase in intracellular AdoMet levels, although this latter effect has only been tested in COS-7 cells.3 Regardless of whether these increases in intracellular AdoMet affect the measurement of half-lives of other enzymes, the effects shown here suggest that the true intracellular half-life of AdoMetDC may be longer than the half-life typically measured by the above techniques, because the intracellular AdoMet concentration is likely to affect the measured half-life. Whether other intracellular factors may alter the susceptibility of AdoMetDC to transamination and/or degradation at a particular AdoMet concentration still remains to be determined.

The current results indicate that transamination of AdoMetDC accelerates its degradation. Assuming that ubiquitination is indeed the rate-limiting step in proteolytic targeting of AdoMetDC, and based on the evidence that ubiquitination can occur on an N-terminal amine group as well as on side chain lysines (52), an intriguing idea is that conversion of the non-amine containing pyruvyl residue to an N-terminal alanine could directly serve as a ubiquitination site. Alternately, in mammalian cells alanine has been shown to be a destabilizing residue in the N-end rule demonstrated by Varshavsky and co-workers (53), so pyruvyl transamination to alanine could create a destabilizing N-terminal amino acid. However, the pyruvyl residue, as well as the cysteine active site residue, which may be alkylated as part of the transamination process (36), are located in an internal cleft of the enzyme (41), and thus unlikely to serve as surface-accessible signals or sites for ubiquitination. It seems more likely that transamination/alkylation accelerates the overall degradation rate by altering the structure of the enzyme in such a way as to expose an internal targeting signal or ubiquitination site, which is then recognized by the ubiquitin conjugation machinery. The overall structure of the active AdoMetDC αβ-dimer is that of a clamshell, and the active site is formed at one end of the generally hydrophobic interface of the two clamshell halves. In addition to the pyruvyl residue and the active site cysteine 82, the other end of this interface (closer to the “hinge” region) is the binding site for putrescine, which both activates the enzyme and stabilizes it against intracellular degradation. One possible mechanism of transamination-dependent acceleration of degradation is that transamination causes increased opening of the clamshell structure, exposing normally buried hydrophobic regions of the protein, which can serve as triggers of rapid degradation (12). Conversely, putrescine binding could be stabilizing AdoMetDC by stabilizing the closed configuration of the clamshell and preventing this exposure. (It is also theoretically possible that putrescine could stabilize the enzyme by preventing or slowing the transamination reaction; however, we have determined that putrescine does not affect the in vitro rate of transamination of the rabbit reticulocyte-expressed human AdoMetDC enzyme at either high or low AdoMet concentrations, suggesting that putrescine is acting to stabilize the enzyme against degradation at some point beyond the transamination step). Current experiments are investigating the ubiquitination site of AdoMetDC, and these results will help clarify potential mechanisms of transamination-accelerated degradation of AdoMetDC.

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