Role of the Carboxyl Terminal MATEE Sequence of Spermidine/Spermine N^1-Acetyltransferase in the Activity and Stabilization by the Polyamine Analog N^1,N^{12}-Bis(ethyl)spermine†

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ABSTRACT: Purified recombinant spermidine/spermine N^1-acetyltransferase (SSAT) was found to be unstable in the absence of polyamines, but the loss of activity could be prevented or reversed by the addition of the polyamine analog and potential antitumor agent N^1,N^{12}-bis(ethyl)spermine (BE-3-4-3), which is known to be a potent inducer of SSAT in mammalian cells. Addition of BE-3-4-3 prevented the loss of SSAT activity and the digestion of the protein by the proteases trypsin, Lys-C, or Glu-C. In the absence of BE-3-4-3, this digestion occurred at the sequence $Lys^{141}Arg^{142}Arg^{143}$ for trypsin or Lys-C and at the sequence $Glu^{151}Glu^{152}$ for Glu-C. When these sites were altered by mutation to residues which are not substrates for these proteases, cleavage in the absence of BE-3-4-3 occurred at residues Lys^{161}, Lys^{166}, and Glu^{162}. These results indicate that the structure of SSAT contains a region that binds to the polyamine analog, BE-3-4-3, and that binding alters the configuration of the protein to prevent protease access to the region from amino acid residue 141 to the carboxyl terminal end (residue 171) of the SSAT. In order to determine the nature of the regulatory sites, specific mutations were made in the SSAT amino acid sequence, and the activity of the resulting SSAT protein and the sensitivity to proteases in the presence and absence of BE-3-4-3 was determined. The results indicate that the carboxyl terminal domain, MATEE, is critical for activity and for protection by BE-3-4-3. Deletion of the five amino acids at the carboxyl terminal increased the apparent K_m for spermidine by 40-fold, reduced the maximal activity by 90%, and abolished protection by BE-3-4-3. Residue Glu^{152} was also important in the activity and protection by BE-3-4-3 since its conversion to Gln or Lys removed the response to BE-3-4-3 and increased the K_m by 4- or 9-fold, respectively. These results suggest that BE-3-4-3 binds to SSAT via an interaction with Glu^{152} and the MATEE sequence at residues 167−171 and that this binding leads to a conformational change that increases the affinity for the polyamine substrate and protects the protein from protease digestion. This effect may play a major role in the increased SSAT activity in cells treated with BE-3-4-3 and related compounds.

Spermidine/spermine N^1-acetyltransferase (SSAT[1]) plays a key role in the degradation and excretion of polyamines in mammalian cells (Pegg, 1986, 1988; Seiler, 1987; Wallace, 1987; Casero & Pegg, 1993). Exposure to high levels of polyamines produces an increase in the amount of SSAT activity (Persson & Pegg, 1984; Pegg & Erwin, 1985; Erwin & Pegg, 1986). The SSAT then acts on intracellular spermidine and spermine. The products of this reaction, N^1-acetylspermidine and N^1-acetylspermine, are rapidly degraded by polyamine oxidase and converted to putrescine (Pegg, 1986; Seiler, 1987). Both putrescine and N^1-acetylspermidine are excreted from the cell (Pegg et al., 1989, 1990). The effect of the induction of SSAT is therefore to reduce the content of spermidine and spermine, and SSAT acts as a homeostatic mechanism to prevent the accumulation of polyamines to high levels which cause cytotoxic effects. A wide variety of stimuli including a number of toxic agents lead to an increase in SSAT activity (Matsui et al., 1981; Casero & Pegg, 1993), and it has been suggested that a common feature of these stimuli is a rise in the free intracellular polyamine content (Pegg, 1986).

Some polyamine analogs such as N^1,N^{11}-bis(ethyl)norspermine (BE-3-3-3), N^1,N^{12}-bis(ethyl)spermine (BE-3-4-3), N,N'-bis[3-(ethylamino)propyl]-1,7-heptanediamine, and a number of related unsymmetrical compounds are very powerful inducers of SSAT levels and are much more active in this induction than the natural polyamines themselves (Casero et al., 1989a, 1995; Saab et al., 1993; Porter et al., 1993; Shappell et al., 1993; Bergeron et al., 1994). They are therefore useful model compounds for studying the mechanism by which SSAT is increased. In addition, these compounds are currently undergoing clinical trials as antitumor agents (Bergeron et al., 1989; Bernacki et al., 1992; Edwards et al., 1990; Marton & Pegg, 1995). Their ability

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[1] Abbreviations: SSAT, spermidine/spermine N^1-acetyltransferase; BE-3-4-3, N^1,N^{12}-bis(ethyl)spermine; bp, base pair; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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to increase SSAT and hence to deplete the cell of the normal polyamines may play a critical role in their ability to kill tumor cells (Casero et al., 1989a,b, 1992; Davidson et al., 1993; Casero & Pegg, 1993; Porter et al., 1993). A better understanding of their mechanism of action may aid in the use of these analogs and the design of improved derivatives.

SSAT regulation is complex and involves multiple sites (Casero & Pegg, 1993; Shappell et al., 1993; Parry et al., 1995a). There is good evidence that the content of SSAT mRNA is increased on treatment with BE-3-3-3 or BE-3-4-3 (Shappell et al., 1993; Casero et al., 1991, 1992; Fogel-Petrovic et al., 1993). However, post-transcriptional mechanisms limit SSAT protein accumulation. Thus, when COS-7 cells were transfected with plasmids expressing the SSAT mRNA under the influence of the SV40 promoter, there was a very large increase in the SSAT mRNA content but little change in the amount SSAT protein content (Parry et al., 1995a). When BE-3-4-3 was added to these cells, SSAT protein accumulated rapidly with a several hundred-fold increase within 12 h. This increase was brought about both by an increase in the translation of the SSAT mRNA and by a reduction in the rate of degradation of the SSAT protein (Parry et al., 1995a). The apparent half-life of the SSAT protein was increased by at least 50-fold from about 20 min to more than 12 h when BE-3-4-3 was added. The simplest explanation for this change is that the binding of BE-3-4-3 changes the structure of SSAT, rendering it resistant to degradation.

Little is known about the SSAT enzyme structure. The derived amino acid sequence from the human cDNA indicates that there are 171 amino acids and a subunit M_r of about 20 000 (Casero et al., 1991). The hamster and mouse SSAT enzymes are very similar, with only eight amino acids showing differences with the human sequence (Casero & Pegg, 1993). The SSAT enzyme is probably a tetramer, but the apparent molecular weight of the mammalian SSATs has been reported to be 65 000−80 000: the possibility of a trimer is not totally ruled out. No key residues in the sequence have been identified, and there are no major regions of homology to other proteins, including other acetyltransferases or other enzymes binding acetyl-CoA.

SSAT is normally present in very low amounts in most mammalian cells. Even when the H157 tumor cell line, in which a very large induction occurs, was used, only 120 μg of protein was obtained from 10^9 cells (Casero et al., 1990). Therefore, there has been very little study of the SSAT protein. Recently, the human SSAT protein has been expressed in *Escherichia coli* and purified in large amounts (Parry et al., 1995b). In this paper, we have used the recombinant SSAT to show directly that the binding of BE-3-4-3 alters the structure of the protein and that this changes the activity and the sensitivity to proteases. We have also used site specific mutagenesis and the expression of SSAT protein in a coupled transcription/translation assay to identify key residues in the carboxyl terminal region of the protein which are essential for activity and to study the location of the protease cleavage and residues needed for protection by BE-3-4-3.

MATERIALS AND METHODS

Materials. Oligodeoxynucleotides were synthesized in the Macromolecular Core Facility, Hershey Medical Center, by using a Millipore Expedite 8905 Nucleic Acid Synthesis System. [1-^{14}C]Acetyl-CoA (50 Ci/mol) was purchased from ICN Biochemicals (Costa Mesa, CA). L-[^{35}S]Methionine (translation grade) was from Dupont NEN (Boston, MA), and [α-^{35}S]thio-dATP was obtained from Amersham (Arlington Heights, IL). M13K07 helper bacteriophage, RNasin, TNT T7 coupled reticulocyte lysate translation system, T4 polynucleotide kinase, and *Sph*I restriction endonuclease were obtained from Promega (Madison, WI). The Sculptor *in vitro* mutagenesis system was obtained from Amersham. The Chameleon double-stranded site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Sequenase 2.0, Sequenase Kit, and *Bam*HI restriction endonuclease were purchased from U.S. Biochemical (Cleveland, OH). Ampli*Taq* DNA polymerase was purchased from Perkin-Elmer Cetus (Norwalk, CT). *Vent* DNA polymerase and *Bsp*EI restriction endonuclease were obtained from New England Biolabs (Beverly, MA). DH5αF′ *E. coli* cells, T4 DNA ligase, and *Xho*I restriction endonuclease were purchased from Gibco BRL (Grand Island, NY). Ultrapure deoxynucleotides were purchased from Pharmacia (Piscataway, NJ). Qiagen plasmid purification columns, and Qiaquick PCR purification and Qiaquick gel extraction kits were purchased from Qiagen Inc. (Chatsworth, CA). Purified trypsin from bovine pancreas (sequencing grade), Glu-C endoproteinase from *Staphylococcus aureus* strain V8 (sequencing grade), azoalbumin, and ampicillin were obtained from the Sigma Chemical Co. (St. Louis, MO). Lys-C endoproteinase from *Lysobacter enzymogenes* (sequencing grade) was purchased from Boehringer Mannheim (Indianapolis, IN).

Preparation of Mutant cDNAs. The pSAT9.3 plasmid containing the SSAT cDNA in the Bluescript vector (Casero et al., 1991) was used as a double-stranded DNA template for site-directed mutagenesis and for the preparation of single-stranded DNA and as a template for amplification by PCR.

All of the carboxyl region deletion mutants were prepared by PCR using a sense primer corresponding to the T7 RNA polymerase promoter region and an antisense primer that was designed to alter a selected codon to an ochre stop codon followed downstream by a *Bam*HI restriction site for ligation.

Point mutations (K161A, M167A, T169A, E170Q, and E171Q) and the double mutation E170K/E171K in the carboxyl terminal region were introduced by PCR using the T7 sense primer and an antisense primer that altered the selected codon(s) and also introduced a *Bam*HI site downstream of the normal stop codon. The two-amino acid extension mutant −172K/173K was prepared by substitution of the normal stop with two lysine codons followed by an ochre stop codon and a downstream *Bam*HI site for ligation. In addition, both E58Q and E62Q mutations were introduced by PCR using the T7 sense primer and antisense primers that each contained the desired mismatch to change Glu to Gln and included the downstream unique *Bsp*EI restriction site.

The products from the PCR were purified using Qiaquick columns prior to digestion with *Xho*I and *Bsp*E1, *Sph*I and *Bam*HI, or *Bsp*E1 and *Bam*HI. The desired fragments were purified after agarose gel electrophoresis using Qiaquick gel extraction columns according to the manufacturer's directions. These fragments were then ligated into a wild type vector cut with the same restriction enzymes.

Other point mutations in SSAT (E23Q, E28Q, E31Q, E32Q, E43Q, E48Q, E58Q, E67Q, E151Q, E152Q, and E152K), the double mutant E151Q/E152Q, and the three-

amino acid mutant K141S/R142A/R143S were prepared using either a ssDNA template as needed for the Sculptor *in vitro* site-directed mutagenesis protocol or a dsDNA template as required for the Chameleon protocol according to the respective manufacturer's directions.

The SSATf1 mutant was made by the deletion of a single base causing a frame shift at the codon-170, changing the amino acid sequence from position 170 onward from EE to RSEDPLVLERPPPRWSSSFCSL, thus extending the 171-residue SSAT by an additional 20 amino acids. The SSATf2 mutant, sequence arose from a PCR error in the synthesis of the K161A mutant resulting in the additional alterations from position 167 of RQQRSEDPLVLERPPPRWSSSFCSL instead of MATEE. The double-stranded plasmid DNA formed in the mutagenesis and PCR product ligation reactions was introduced into the *E. coli* strain DH5αF′ by electroporation (Stanley & Pegg, 1991). Minipreparations of DNA were prepared from selected colonies and screened for the presence of the desired mutation by sequencing. Plasmid DNA from confirmed mutants was prepared from 500 mL cultures using alkaline lysis and purification on Qiagen-Tip 500 anion exchange columns. The sequence of the entire coding region of the SSAT mutants was checked to verify the absence of secondary mutations.

Expression of Wild Type and Mutant SSATs in Vitro from Plasmid DNA. Transcription/translation of the wild type and mutant pSAT9.3 plasmids was carried out using the Promega TNT coupled reticulocyte lysate system using a minor modification of the manufacturer's directions; for most assays, a total volume of 12.5 μL containing 0.125 μg of plasmid DNA was used (in some experiments, a larger volume with the same ratio of plasmid DNA was used). After a 45 min incubation at 30 °C to synthesize the SSAT protein, aliquots were removed for the determination of SSAT activity. In order to determine the relative levels of wild type and mutant SSAT synthesized, translation assays containing [^{35}S]methionine were conducted in parallel and the labeled proteins separated by SDS−PAGE according to the method of Laemmli (1970).

Purification of Recombinant SSAT from E. coli. Recombinant human SSAT was expressed in the *E. coli* strain DH5α from the pINSAT2 vector and purified to homogeneity in a final step from a *sym*-norspermidine−sepharose affinity column as described previously (Parry et al., 1995a).

Protease Digestion of Recombinant and TNT-Synthesized SSAT Protein. BE-3-4-3 was prepared as a stock solution in buffer A (50 mM Tris-HCl (pH 7.5), 2.5 mM DTT, and 0.1 mM EDTA). Trypsin was prepared as a 10 ng/μL stock solution in 1 mM HCl, Lys-C as a 10 or 100 ng/μL solution in water, and Glu-C as a 100 ng/μL solution in water. Reaction mixtures containing 0.5 ng of purified SSAT protein were incubated in the presence or absence of proteases (up to 5 ng) and in the presence or absence of 2.5 μM BE-3-4-3 in a total volume of 20 μL in buffer A at 30 °C. SSAT activity was determined at indicated times as described below. Wild type and mutant SSAT proteins were synthesized in a standard 12.5 μL TNT reaction described above. Aliquots of 2 μL (unlabeled) or 5 μL (^{35}S-labeled) SSAT protein were removed for subsequent endoproteinase digestion in a final reaction volume of 20 μL (unlabeled) or 50 μL (^{35}S-labeled) in buffer A. Each 20 μL reaction mixture was digested with either 5−50 ng of trypsin, 10−500 ng of Lys-C, or 10−500 ng of Glu-C for up to 1 h at 30 °C, and the entire reaction mixture was assayed for remaining SSAT activity. Each 50 μL reaction containing ^{35}S-labeled protein was digested with either 125 ng of trypsin, 125 ng of Lys-C, or 1.25 μg of Glu-C under the same conditions, stopped at given times by the addition of SDS sample buffer, and separated by SDS−PAGE.

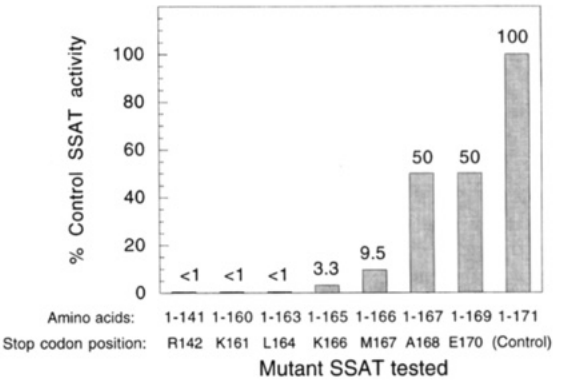

FIGURE 1: Effect of truncation of the carboxyl end of SSAT on activity. Stop codons were introduced at positions 142, 161, 164, 167, 168, 166, and 170 as indicated. SSAT protein was produced in the coupled transcription/translation system, and the SSAT activity was measured in the standard assay with 3 mM spermidine and 16 μM [^{14}C]acetyl-CoA as substrates. Results were expressed as the percentage of the activity of the control SSAT which contains 171 amino acids. These percentage values are shown above the bars, and the number of amino acids remaining and the residue whose codon was converted to a stop codon are shown below. The experiment was repeated at least three times with identical results.

Assay for SSAT Activity. All dilutions of purified recombinant protein or TNT-synthesized proteins were in buffer A. SSAT activity was measured by assaying the conversion of [1-^{14}C]acetyl-CoA into [1-^{14}C]acetylspermidine at 30 °C for 10 min as described previously (Matsui et al., 1981). A standard assay mixture contained 50 mM Tris-HCl (pH 7.8), 3 mM spermidine, and 16 μM [1-^{14}C]acetyl-CoA (50 mCi/mmol) in a total volume of 100 μL.

RESULTS

Effects of Mutations on SSAT Activity. Mutations were produced in pSAT9.3 which contains the SSAT coding sequence and 145 bp of 5′UTR and 123 bp of 3′UTR in a Bluescript vector. The effects of these changes in the SSAT amino acid sequence could readily be investigated by expression of the mutated cDNAs in a coupled transcription/translation system and measurement of the amount of SSAT activity produced. The extent of SSAT protein synthesis was determined by carrying out the transcription/translation reaction in the presence of [^{35}S]methionine, separating the labeled SSAT protein by SDS−PAGE, and densitometrically analyzing the band corresponding to the SSAT protein. All results were corrected for the extent of SSAT synthesis, taking into account the fact that some of the mutants examined have one less methionine residue than the wild type protein. However, such correction for the extent of synthesis made only a small change in the results since all mutants were expressed to levels close (87−131%) to wild type [except for R142Stop (46% expression) and K161Stop (68% expression) which are completely inactive].

The effects of progressive deletions from the carboxyl terminal of the protein are shown in Figure 1. Deletion of the carboxyl terminal six amino acids (KMATEE) from SSAT led to virtually a complete loss of activity (Figure 1). The removal of only two terminal Glu residues reduced activity by 50%, and most of the rest of the activity was

Table 1: Effect of Mutations in Different Domains of SSAT on SSAT Activity

mutation	SSAT activity[a]	apparent K_m for spermidine (μM)[a]	BE-3-4-3 stabilization against trypsin[a,b]
none	100	70	yes
mutations in the carboxyl domain			
K161A	74	ND	yes
K166A	134	ND	yes
M167A	45	288	partial (38%)
M167Stop	9	2740	no
T169A	86	ND	yes
E170Q	62	70	yes
E171Q	95	63	yes
E170Stop	50	101	partial (45%)
E170K/E171K	49	150	no
−172K/173K	57	113	yes
mutations in the central domain			
E151Q	105	157	yes
E152Q	70	301	no
E152K	10	644	no
E151Q/E152Q	36	357	no
mutations in the amino terminal domain			
E23Q	93	ND	yes
E28Q	45	210	yes
E31Q	72	ND	yes
E32Q	114	ND	yes
E43Q	80	ND	yes
E48Q	96	ND	yes
E58Q	75	ND	yes
E62Q	92	ND	yes
E67Q	95	73	yes

[a] The results shown are from representative experiments which were repeated three times with results which agreed within ±20% of the value shown. [b] Yes indicates that more than 80% of the SSAT protein remained undigested when incubated with trypsin for 1 h in the presence of BE-3-4-3. No indicates that less than 15% remained unprotected under the same conditions. ND, not determined.

removed when Met[167] was deleted. These results are confirmed by point mutations in the KMATEE region shown in Table 1. Conversion of Thr[169] or Lys[166] to Ala or conversion of Glu[171] to Gln produced little or no reduction in SSAT activity. However, alteration of the Glu[170] to Gln reduced activity by 38%, and conversion of Met[167] to Ala reduced activity by 55%. The double change of the two terminal Glu residues to Lys reduced activity by 51%, and a 43% reduction was produced by addition of two Lys residues to the carboxyl end forming residues 172 and 173.

All of these assays were carried out with a standard assay system that has a very high spermidine level of 3 mM. This is more than 40 times the K_m, and it is possible that mutants with a greatly reduced binding efficiency for the substrate would still give a substantial amount of activity in this assay. The apparent K_m values were therefore measured for the most critical mutants (Table 1). Although such measurements in crude extracts may be influenced by other substances present in the assay, the apparent K_m for the control SSAT was 70 μM which agrees with that found with the purified human enzyme from H157 cells (Casero et al., 1990) and the recombinant human SSAT (Parry et al., 1995b). The M167A mutant showed a 5-fold increase in the apparent K_m, and the M167Stop mutant gave a very large increase of about 40-fold. This suggests that the carboxyl domain is involved in the polyamine substrate binding.

Another set of interesting mutants was made by mutations in the Glu residues at positions 151 and 152. Changing both of these to Gln reduced activity by 64% in the standard assay

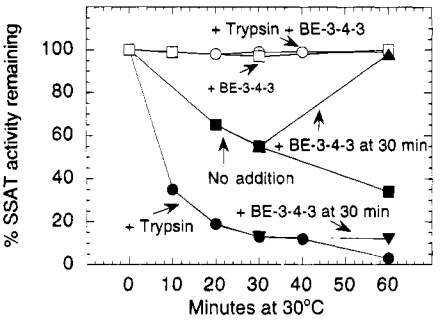

FIGURE 2: Effect of trypsin and BE-3-4-3 on activity of SSAT. Reaction mixtures containing 0.5 ng of purified recombinant SSAT in acetylase buffer A were incubated at 30 °C in the absence or presence of 2.5 ng of trypsin and in the absence or presence of 2.5 μM BE-3-4-3 (the latter added either at the start of the incubation or after 30 min as shown). SSAT activity was determined at the times indicated. The results shown are the mean of triplicate observations which agreed within ±10%.

and increased the apparent K_m 5-fold (Table 1). The individual mutations E151Q and E152Q increased the K_m by 2- and 4-fold, respectively, but only the change of E152Q affected the activity in the standard assay (by 30%). However, the conversion of Glu[152] to a positively charged Lys residue reduced activity by 90% and increased the K_m 9-fold. These results suggest that Glu[152] is also involved in substrate binding.

Changes of individual Glu residues to Gln in the amino terminal domain of the SSAT had little effect on the activity except for the change of E28Q which reduced activity by 55% and increased the K_m about 3-fold (Table 1).

Stabilization of Purified SSAT by BE-3-4-3. The purified SSAT protein was very unstable when stored or incubated in the absence of polyamines. Furthermore, addition of a polyamine analog such as BE-3-4-3 had a biphasic effect on SSAT activity. Addition of concentrations of BE-3-4-3 up to 5 μM increased the SSAT activity by up to 70%, depending on the SSAT preparation, whereas higher concentrations were inhibitory (results not shown). These results suggest that there are two binding sites for polyamines or analogs on SSAT, the active site and a regulatory site. Binding to the latter would change the configuration of the SSAT protein to enhance activity.

In order to test this, the purified SSAT was incubated in the presence or absence of 2.5 μM BE-3-4-3 and in the presence or absence of trypsin (Figure 2). When incubated without protease in the absence of BE-3-4-3, activity was lost quite rapidly. This loss was completely prevented by the addition of BE-3-4-3 and was reversed when BE-3-4-3 was added after 30 min. When trypsin was added, activity was lost even more rapidly, but this loss was also completely prevented by BE-3-4-3. The loss of activity in response to 30 min exposure to trypsin was not reversed by the addition of BE-3-4-3, but the activity remaining at 30 min was stabilized. These results are not due to the inactivation of trypsin by BE-3-4-3 since there was no effect on the degradation of azoalbumin by trypsin in the presence of up to 3 mM BE-3-4-3 (results not shown). These results suggest that the binding of BE-3-4-3 to the regulatory site converts the SSAT protein to a form that is resistant to trypsin digestion.

Similar experiments (not shown) established that, in the absence of a polyamine analog, the SSAT was rapidly inactivated by proteases Lys-C or Glu-C but that the SSAT

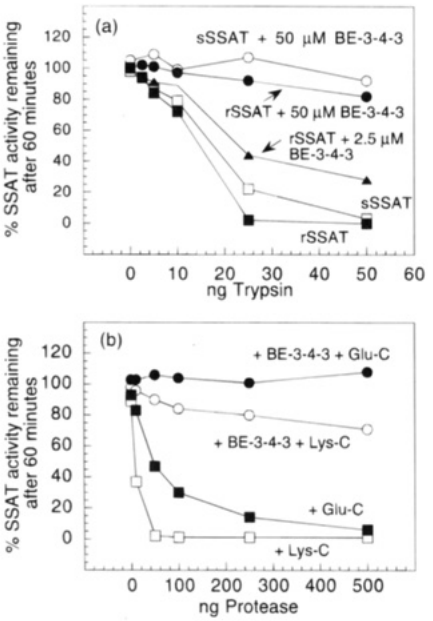

FIGURE 3: Effect of BE-3-4-3 on the loss of SSAT activity produced by proteases. Panel (a) shows the effects of trypsin on SSAT activity. rSSAT indicates that the purified recombinant human SSAT was used. sSSAT indicates that protein synthesized from plasmid in a coupled transcription/translation reaction during a 45 min incubation at 30 °C was used. Aliquots (2 μL) of the synthesis reaction mixture containing sSSAT were taken for digestion with increasing amounts of trypsin for 1 h at 30 °C in the absence or presence of 50 μM BE-3-4-3 and then assayed for remaining SSAT activity. A parallel experiment was carried out in which 0.4 ng of rSSAT was substituted for plasmid in a lysate mixture and otherwise treated identically with trypsin in the absence or presence of 2.5 or 50 μM BE-3-4-3 and assayed for SSAT activity. Panel (b) shows the effects of Lys-C and of Glu-C on sSSAT activity. sSSAT was synthesized as described for panel (a), and identical aliquots were taken for digestion with increasing amounts of protease for 1 h at 30 °C in the absence or presence of 50 μM BE-3-4-3. The SSAT activity remaining was determined after this time. The results shown are the mean of triplicate observations which agreed within ±10%.

was completely protected by 2.5 μM BE-3-4-3 in the presence of 5 ng of Lys-C or Glu-C for 1 h.

Stabilization of SSAT Produced in the Transcription/Translation System by BE-3-4-3. In order to examine the effect of mutations in the SSAT sequence on the response to BE-3-4-3, it was necessary to carry out experiments with trypsin and other proteases on the labeled SSAT synthesized in the transcription/translation system. All of the control and the mutant SSAT proteins formed in this system were stable in the absence of protease addition. As shown in Figure 3a, the SSAT protein formed in this system was degraded by trypsin and this degradation was totally prevented by the addition of 50 μM BE-3-4-3. The sensitivity of the synthesized SSAT to trypsin was similar to that of purified SSAT added to an identical transcription/translation assay tube which had not had the pSAT9.3 plasmid added (Figure 3a). It was necessary to add ≥25 μM BE-3-4-3 to get a maximal protective effect in this system. As shown in Figure 3a, 2.5 μM, which was sufficient to give complete protection in the studies with the purified SSAT protein (Figure 2), gave only partial protection. This may be due to the presence of other components such as ribosomes that may bind BE-3-4-3. The presence of BE-3-4-3 also protected the synthesized SSAT from degradation by the proteases Glu-C and Lys-C (Figure 3b).

Site of Protease Cleavage of SSAT. The time course of the digestion of SSAT protein by trypsin showed that the

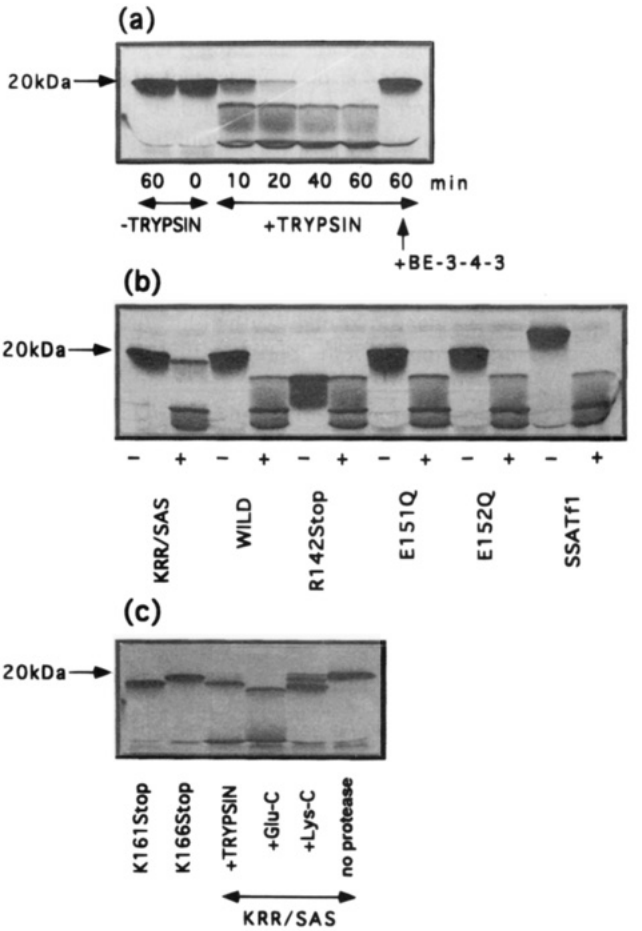

FIGURE 4: Fragments generated by proteases from SSAT and mutant SSAT proteins. Wild type and selected mutant SSAT plasmids were used to synthesize the corresponding ^{35}S-labeled protein in the coupled transcription/translation system as described in the Materials and Methods. Aliquots were taken for protease digestion as described for each panel and the resulting labeled fragments detected following separation by SDS−PAGE using 15% polyacrylamide gels. The position of a M_r 20 kDa protein corresponding to undigested wild type SSAT is indicated by the arrow. Panel (a) shows a time course of the digestion of wild type SSAT at 30 °C with 125 ng of trypsin in the absence or presence of 50 μM BE-3-4-3. Panel (b) shows the fragments produced after treatment of wild type and mutant [K141S/R142A/R143S (indicated as KRR/SAS), R142Stop, E151Q, E152Q, and SSATf1] SSAT proteins without (−) or with (+) 125 ng of trypsin for 1 h at 30 °C. Panel (c) shows the fragments generated following digestion of the KRR/SAS mutant SSAT protein with either 125 ng of trypsin, 1.25 μg of Glu-C, or 125 ng of Lys-C. The K161Stop and K166Stop truncated proteins are included for fragment size comparison

loss of activity was accompanied by the appearance of a degradation product with a M_r of about 16 kDa (Figure 4a). This fragment must be derived from the amino terminal portion of the SSAT since the same fragment was produced when two mutants which had carboxyl terminal extensions of 20 amino acids [SSATf1 (Figure 4b) and SSATf2 (results not shown)] were used. A fragment of the correct size would be produced by cutting at the sequence $Lys^{141}Arg^{142}Arg^{143}$, and the mutant R142Stop SSAT protein had a mobility identical to the above-mentioned 16 kDa tryptic fragment (Figure 4b). Furthermore, when the $Lys^{141}Arg^{142}Arg^{143}$ sequence was changed to $Ser^{141}Ala^{142}Ser^{143}$, this fragment was not seen after trypsin or Lys-C digestion (Figure 4b,c). These results establish that the primary site of SSAT digestion with trypsin is at positions 141−143. Digestion with Lys-C gave a band of 16 kDa identical to that found

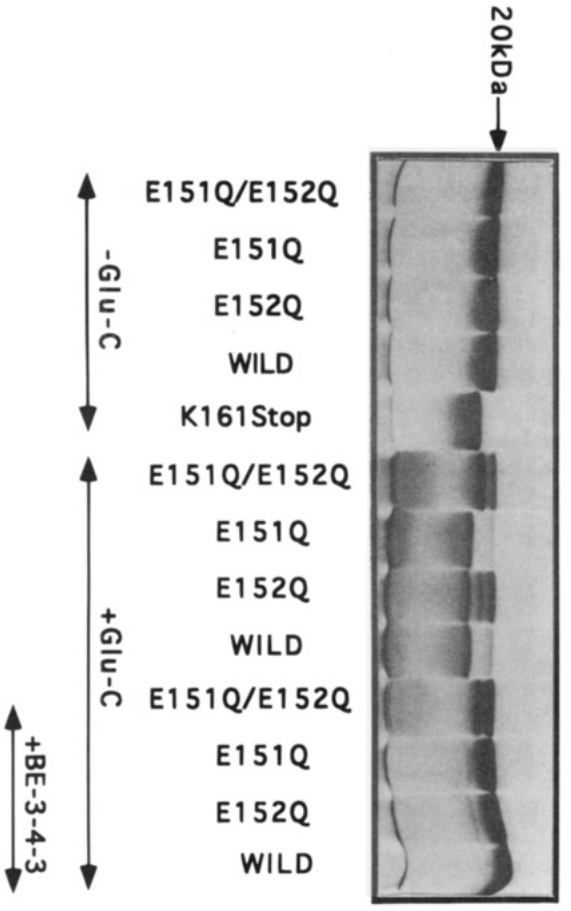

FIGURE 5: Size of Glu-C digestion products from control, E151Q/E152Q, E151Q, and E152Q mutant SSAT proteins. The ^{35}S-labeled proteins shown were prepared as described in the legend to Figure 4. Aliquots (5 μL) of the synthesized proteins were then incubated in the absence or presence of 1.25 μg of Glu-C and in the absence or presence of 50 μM BE-3-4-3 as indicated. After a 1 h incubation at 30 °C, the digested products were separated by SDS−PAGE. The K161Stop SSAT truncated protein is included as a marker for fragment size comparison.

with trypsin when acting on the control SSAT (results not shown), indicating that Lys-C cuts at Lys^{141}.

The K141S/R142A/R143S mutant SSAT was still sensitive to digestion by trypsin or Lys-C, giving two larger bands that had M_r values of about 18.7 and 19.4 kDa. The predominant product with trypsin had a mobility almost identical to that of the mutant K161Stop (Figure 4c), indicating that Lys^{161} is also a site for tryptic cleavage. Digestion of this mutant SSAT with Lys-C gave two bands consistent with cleavage at both Lys^{161} and Lys^{166} (Figure 4c). These sites are less susceptible than the sites at positions 141−143 since these cleavage products were not seen with the control SSAT protein even at early stages of digestion (Figure 4a).

The K141S/R142A/R143S mutant SSAT was degraded by Glu-C in a manner similar to that of the control SSAT (Figures 4c and 5), indicating that this mutation does not produce a general distortion in the protein structure. Treatment of wild type SSAT protein with Glu-C produced a fragment having a mobility corresponding to a M_r of about 17.4 kDa (Figure 5). This suggests a primary cleavage site at either Glu^{151} or Glu^{152}. In order to test this, mutations were made in this region of the protein. Digestion of a E151Q mutant occurred as readily as with control SSAT and resulted in a fragment of the same size, suggesting that E151 is not the primary cleavage site (Figure 5). The E152Q

SSAT Cleavage Positions

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     1        10        20        30        40        50
1    MAKFVIRPAT AADCSDILRL IKELAKYEYM EEQVILTEKD LLEDGFGEHP  50
51   FYHCLVAEVP KEHWTPEGHS IVGFAMYYFT YDPWIGKLLY LEDFFVMSDY 100
101  RGFGIGSEIL KNLSQVAMRC RCSSMHFLVA EWNEPSINFY KRRGASDLSS 150
                                              Trypsin/Lys-C (1)
Glu-C (2)   Glu-C (2)
151  EEGWRLFKID KEYLLKMATE E                              171
Glu-C (1)   Trypsin/Lys-C (2)
```

FIGURE 6: Positions of SSAT cleavage by proteases. The primary sites of cleavage by trypsin, Lys-C, and Glu-C are double-underlined and marked with (1). The secondary sites at which the protein is degraded if the primary site is mutated are double-underlined and shown as (2). The carboxyl terminal sequence essential for activity is shown in bold italic characters.

mutant appeared to be more resistant to degradation by Glu-C than either the wild type or E151Q proteins, with at least 20% of the undigested protein remaining under the same conditions. Two large degradation products from this mutant were produced (Figure 5). The larger product was slightly larger than the K161Stop mutant SSAT used as a marker, suggesting cleavage at Glu^{162}, while the second degradation product corresponded to cleavage at Glu^{151}. When both Glu residues at positions 151 and 152 were substituted with Gln, the mutant SSAT protein showed a resistance to digestion with Glu-C similar to that seen with the E152Q mutant, but only the fragment cleaved at Glu^{162} was produced (Figure 5). These results indicate that, for wild type SSAT, Glu^{152} is the primary cleavage site for Glu-C digestion and that Glu^{151} and Glu^{162} become the cleavage sites when Glu^{152} is mutated to Gln^{152}.

A summary of the cleavage sites of SSAT is shown in Figure 6. Since all of these reactions remove the carboxyl terminal domain, which is shown in Figure 1 to be essential for activity, such cleavage is likely to lead to SSAT inactivation and to account for the loss of activity in the presence of these proteases.

Effect of Mutations on the Ability of BE-3-4-3 To Protect the SSAT Protein from Protease Digestion. The presence of BE-3-4-3 completely prevented protease digestion of SSAT with 125 ng of trypsin (Figures 4a, 7a, and 8a), 1.25 μg of Glu-C (Figure 7b), or 125 ng of Lys-C (results not shown). In order to investigate which amino acid residues were involved in the interaction leading to this protection, the mutant SSAT proteins were tested for protection by BE-3-4-3 against protease degradation. A comparison of the mutants tested for protection against trypsin in this way is shown in the right column of Table 1. These results show that the MATEE region and Glu^{152} are involved in this protection.

The importance of the carboxyl terminal domain in this protection is demonstrated by the experiment shown in Figure 7 where the M167Stop and A168Stop mutations prevented the protection by BE-3-4-3 against either trypsin (Figure 7a) or Glu-C (Figure 7b). The M167A mutation clearly reduced the protection but did not abolish it completely. Similarly, as shown in Figure 8b, the E170Stop mutant reduced the protection against trypsin degradation, whereas neither the E170Q nor the E171Q mutant SSAT proteins were greatly different from the control.

The E152Q and E151Q/E152Q mutant SSATs were not protected from tryptic digestion by BE-3-4-3, whereas E151Q was totally protected (Figure 8a). This indicates that SSAT

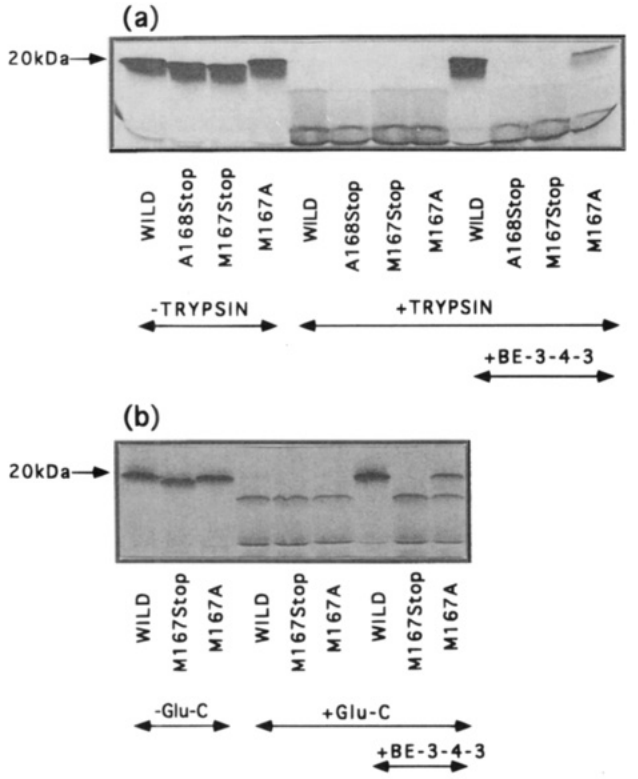

FIGURE 7: Effect of M167Stop, M167A, and A168Stop mutations on BE-3-4-3-mediated protection of SSAT from proteases. The ^{35}S-labeled proteins shown were prepared and subjected to protease digestion and SDS−PAGE as described in the legends to Figures 4 and 5. Panel (a) compares the ability of BE-3-4-3 to protect wild type and mutant SSAT proteins from tryptic digestion. Panel (b) compares the ability of BE-3-4-3 to protect wild type and mutant SSAT proteins from Glu-C digestion.

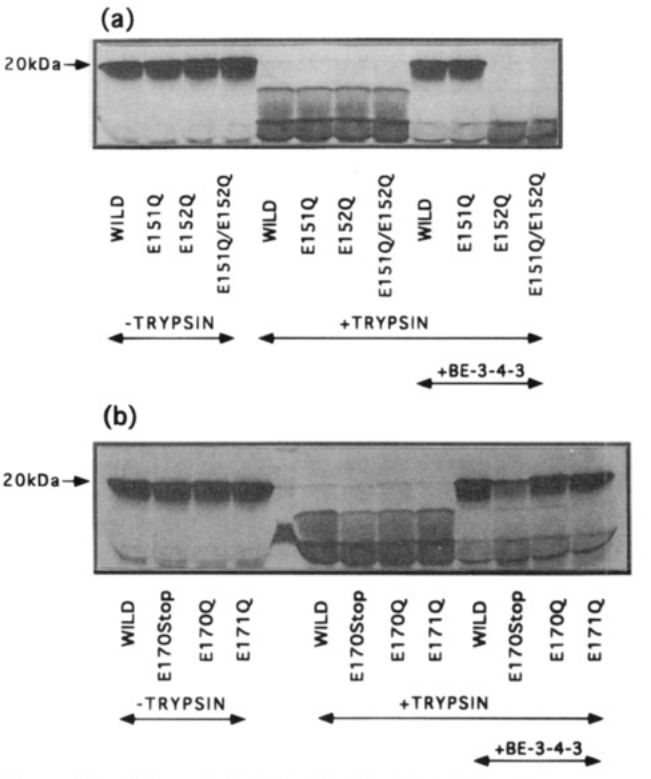

FIGURE 8: Effect of E151Q, E152Q, E151Q/E152Q, E170Q, E171Q, and E170Stop on BE-3-4-3-mediated protection of SSAT from trypsin. The ^{35}S-labeled proteins shown were prepared and subjected to protease digestion and SDS−PAGE as described in the legend to Figures 4. Panel (a) shows the effects of single and double mutations at Glu^{151} and Glu^{152} and panel (b) shows the effects of point mutations as well as the truncation of the terminal two residues (Glu^{170} and Glu^{171}).

residue Glu^{152} but not Glu^{151} is essential for protection against trypsin by BE-3-4-3.

DISCUSSION

Polyamines must bind at the active site of SSAT for acetylation to take place, and previous studies showing that polyamine analogs such as BE-3-4-3 and BE-3-3-3 act as weak competitive inhibitors of the acetylation of spermine indicate that these analogs must also bind at the active site (Pegg et al., 1990; Libby et al., 1991). The results presented in this paper support the concept [also suggested by Wallace and Quick (1994)] that SSAT also contains a regulatory binding site for polyamine analogs or polyamines and that binding to this site changes the configuration of the protein to increase activity and to protect the protein from rapid degradation. The ability of either the natural polyamines or analogs to increase the half-life of SSAT which has a very short half-life in the absence of these compounds has been documented in several studies (Matsui & Pegg, 1981; Erwin & Pegg, 1986; Persson & Pegg, 1994; Libby et al., 1989; Pegg et al., 1990; Porter et al., 1991).

In the presence of BE-3-4-3, the SSAT protein is highly resistant to proteases, but in the absence of the analog, the protein is readily cleaved in the region from amino acid 141 to 152 and sites from 160 to 166 are also susceptible (see Figure 6). The configurational change is therefore likely to prevent the access of the proteases to these regions. Studies with the mutant SSAT proteins suggest that Glu^{152} and the C terminal MATEE region are essential for BE-3-4-3 to bring about this protection. Changing either Glu^{152} or the two terminal Glu^{170} and Glu^{171} residues to Lys (mutants E152K and E170K/E171K) abolishes all protection by BE-3-4-3 (Table 1). A plausible explanation for these observations is therefore that interactions between the positively charged BE-3-4-3 and the acidic Glu side chains are needed to cause the configurational change in SSAT that restricts access of the proteases to residues in the region of amino acids 141 to 166. However, other parts of the MATEE region must also contribute since the E170Stop mutant can be partially protected by BE-3-4-3. The Met^{167} residue is likely to be the other critical site since mutant M167A is also only partially protected (see Table 1).

The only mutations studied in the present work affecting SSAT activity substantially when assayed under the standard conditions were changes involving Glu^{28}, Glu^{152}, and the carboxyl terminal MATEE region. It is possible that Glu^{28} forms part of the active site, but it does not appear to be involved in the stabilization by BE-3-4-3. It is also likely that the region protected from degradation by BE-3-4-3 forms part of the active site since (a) Glu^{152} is likely to be involved since its mutation to Gln (E152Q) or Lys (E152K) causes a 4- and 9-fold increase in the K_m, respectively, and (b) mutation of residue Arg^{143} to Ala leads to a loss of activity and an increase in the K_m for acetyl-CoA (C. S. Coleman and A. E. Pegg, unpublished observations).

The results indicate that the carboxyl terminal MATEE region is of major importance in the active site as well as in mediating the regulatory change in response to polyamine analogs. As shown in Table 1, removal of this sequence leads not only to a loss of the ability of BE-3-4-3 to confer resistance to trypsin but also to a 90% loss in activity and a

40-fold increase in the K_m for spermidine. The data are consistent with an interaction with BE-3-4-3 which places the terminal carboxyl sequence in a configuration that aids in the binding of spermidine to the active site. Analysis of mammalian SSAT by gel filtration indicates that the active enzyme is likely to be a tetramer or possibly a trimer. It is probable that the multimeric form of the enzyme is needed for activity, and it is possible that the key role of the carboxyl end is involved in the formation of the active tetramer.

There are other examples of the critical importance of an extreme carboxyl terminal region of enzymes for activity. A most notable case is that of thymidylate synthase, where the carboxyl terminal residues are essential for activity and folate binding. Studies of the enzyme·substrate complex by X-ray crystallography have shown that folate binding induces a major conformational change in the terminal residues where the terminal Val^{316} moves to anchor a lid that forms over the bound ligands (Carreras et al., 1992; Finer-Moore et al., 1993). Such a mechanism would be consistent with our results on SSAT. Attempts to determine the structure of SSAT in the presence and absence of BE-3-4-3 by X-ray crystallography are currently in progress in collaboration with Drs. N. Campobasso and S. E. Ealick. It should be noted that, at present, we are unable to distinguish between the inability of the E152Q and M167Stop mutants to bind BE-3-4-3 or their inability to undergo the normal conformational change upon binding of the drugs.

Studies with proteins containing residues altered by site-directed mutagenesis can be criticized on the grounds that the alterations introduced may produce a general distortion in the structure of the protein due to incorrect folding not directly related to the alteration in the amino acid side chain. Although this cannot be ruled out entirely, the fact that all of the mutant SSAT proteins tested are expressed at similar levels in the lysates and that the characteristic tryptic digestion pattern with the first cleavage occurs at positions 141−143 suggests that this is not the case.

It should be noted that our experiments using protease sensitivity were designed to evaluate SSAT structure and changes in structure in response to polyamine analogs. The relevance of these changes to the stabilization of SSAT protein in cells remains to be determined. At present, nothing is known of the cellular system responsible for the degradation of SSAT. The mutants described in the present work, particularly E152Q and E170K/E171K, which have substantial SSAT activity but are not converted to a protease resistant form by the binding of BE-3-4-3, can be used to determine the importance that the configurational change in response to the analog has in the control of SSAT protein content and activity. It may also be possible to engineer changes in the SSAT amino acid sequence such that the protein assumes the fully active, protease resistant form even in the absence of polyamine analogs. If such a form of SSAT proves to be stable *in vivo*, it could then be expressed in mammalian cells and used to determine the effects on cellular physiology of SSAT in the absence of polyamine analog inducers. Finally, the assays of the activation of recombinant SSAT and the protection of the enzyme from protease digestion provided by polyamine analogs described in this paper may be useful for evaluation of polyamine analogs for their ability to interact with SSAT and to cause an increase in its activity.

REFERENCES

Bergeron, R. J., Hawthorne, T. R., Vinson, J. R. T., Beck, D. E., & Ingeno, M. J. (1989) *Cancer Res. 49*, 2959−2964.

Bergeron, R. J., McManis, J. S., Liu, C. Z., Feng, Y., Weimar, W. R., Luchetta, G. R., Wu, Q., Ortiz-Ocasio, J., Vinson, J. R. T., Kramer, D., & Porter, C. (1994) *J. Med. Chem. 37*, 3464−3476.

Bernacki, R. J., Bergeron, R. J., & Porter, C. W. (1992) *Cancer Res. 52*, 2424−2430.

Carreras, C. W., Climie, S. C., & Santi, D. V. (1992) *Biochemistry 31*, 6038−6044.

Casero, R. A., & Pegg, A. E. (1993) *FASEB J. 7*, 653−661.

Casero, R. A., Celano, P., Ervin, S. J., Porter, C. W., Bergeron, R. J., & Libby, P. (1989a) *Cancer Res. 49*, 3829−3833.

Casero, R. A., Ervin, S. J., Celano, P., Baylin, S. B., & Bergeron, R. J. (1989b) *Cancer Res. 49*, 639−643.

Casero, R. A., Celano, P., Ervin, S. J., Wiest, L., & Pegg, A. E. (1990) *Biochem. J. 270*, 615−620.

Casero, R. A., Celano, P., Ervin, S. A., Applegren, N. B., Wiest, L., & Pegg, A. E. (1991) *J. Biol. Chem. 266*, 810−814.

Casero, R. A., Mank, A. R., Xiao, L., Smith, J., Bergeron, R. J., & Celano, P. (1992) *Cancer Res. 52*, 5359−5363.

Casero, R. A., Mank, A. R., Saab, N. H., Wu, R., Dyer, W. J., & Woster, P. M. (1995) *Cancer Chemother. Pharmacol. 36*, 69−74.

Davidson, N. E., Mank, A. R., Prestigiacomo, L. J., Bergeron, R. J., & Casero, R. A. (1993) *Cancer Res. 53*, 2071−2075.

Edwards, M. L., Prakash, N. J., Stemerick, D. M., Sunkara, S. P., Bitonti, A. J., Davis, G. F., & Dumont, J. A. (1990) *J. Med. Chem. 33*, 1369−1375.

Erwin, B. G., & Pegg, A. E. (1986) *Biochem. J. 238*, 581−587.

Finer-Moore, J. S., Fauman, E. B., Foster, P. C., Perry, K. M., Santi, D. V., & Stroud, R. M. (1993) *J. Mol. Biol. 232*, 1101−1116.

Fogel-Petrovic, M., Shappell, N. W., Bergeron, R. J., & Porter, C. W. (1993) *J. Biol. Chem. 268*, 19118−19125.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Libby, P. R., Bergeron, R. J., & Porter, C. W. (1989) *Biochem. Pharmacol. 38*, 1435−1442.

Libby, P. R., Ganis, B., Bergeron, R. J., & Porter, C. W. (1991) *Arch. Biochem. Biophys. 284*, 238−244.

Marton, L. J., & Pegg, A. E. (1995) *Annu. Rev. Pharm. 35*, 55−91.

Matsui, I., & Pegg. A. E. (1980) *Biochem. Biophys. Acta 633*, 87−94.

Matsui, I., Wiegand, L., & Pegg, A. E. (1981) *J. Biol. Chem. 256*, 2454−2459.

Parry, L., Balaña Fouce, R., & Pegg, A. E. (1995a) *Biochem. J. 305*, 451−458.

Parry, L., Lopez-Ballester, J., Wiest, L., & Pegg, A. E. (1995b) *Biochemistry 34*, 2701−2709.

Pegg, A. E. (1986) *Biochem. J. 234*, 249−262.

Pegg, A. E. (1988) *Cancer Res. 48*, 759−774.

Pegg, A. E., & Erwin, B. G. (1985) *Biochem. J. 231*, 285−289.

Pegg, A. E., Wechter, R., Pakala, R., & Bergeron, R. J. (1989) *J. Biol. Chem. 264*, 11744−11749.

Pegg, A. E., Pakala, R., & Bergeron, R. J. (1990) *Biochem. J. 267*, 331−338.

Persson, L., & Pegg, A. E. (1984) *J. Biol. Chem. 259*, 12364−12367.

Porter, C. W., Ganis, B., Libby, P. R., & Bergeron, R. J. (1991) *Cancer Res. 51*, 3715−3720.

Porter, C. W., Bernacki, R. J., Miller, J., & Bergeron, R. J. (1993) *Cancer Res. 53*, 581−586.

Saab, N. H., West, E. E., Bieszk, N. C., Preuss, C. V., Mank, A. R., Casero, R. A., & Woster, P. M. (1993) *J. Med. Chem. 36*, 2998−3004.

Seiler, N. (1987) *Can. J. Physiol. Pharmacol. 65*, 2024−2035.

Shappell, N. W., Fogel-Petrovic, M. F., & Porter, C. W. (1993) *FEBS Lett. 321*, 179−183.

Stanley, B. A., & Pegg, A. E. (1991) *J. Biol. Chem. 266*, 18502−18506.

Wallace, H. M. (1987) *Med. Sci. Res.*, 1437−1440.

Wallace, H. M., & Quick, D. M. (1994) *Biochem. Soc. Trans. 22*, 870−875.

BI951263M