RESEARCH COMMUNICATION

Structure and critical residues at the active site of spermidine/spermine-*N*¹-acetyltransferase

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Spermidine/spermine-*N*¹-acetyltransferase (SSAT) is a key enzyme in the degradation of polyamines. Alanine-scanning mutagenesis of all eight arginine residues was used to investigate the arginine residues involved in acetyl-CoA binding. The results indicate that Arg¹⁰¹, Arg¹⁴² and Arg¹⁴³ are important for such binding. The apparent *K*_m values for acetyl-CoA were significantly increased when any one of these residues was replaced by an alanine residue. These mutations also abolished the ability of acetyl-CoA to protect the protein from digestion by trypsin. Co-expression of the inactive R101A (Arg¹⁰¹ → Ala) mutant and an E152K (Glu¹⁵² → Lys) mutant, previously known to inactivate

SSAT, led to restoration of activity, showing that the active enzyme is a dimer with residues contributed by both subunits. The double mutant R101A/E152K acted as a dominant negative when co-expressed with the wild-type SSAT. Transfection of COS-7 cells with a plasmid producing this mutant greatly attenuated the increase in SSAT activity brought about by *N*¹,*N*¹²-bis(ethyl)spermine. These results indicate that the double mutant R101A/E152K-SSAT protein can be used to evaluate the importance of SSAT activity in response to exogenous polyamines or polyamine analogues.

INTRODUCTION

Spermidine/spermine-*N*¹-acetyltransferase (SSAT), which catalyses the conversion of spermidine and spermine into their *N*¹-acetyl derivatives, is strongly induced by polyamines and polyamine analogues [1–3]. This induction is likely to be important in protection of the cells against the toxic effects of excess polyamines, since the acetylated derivatives are substrates for excretion and degradation [4,5]. In contrast, the massive induction of SSAT brought about by polyamine analogues such as *N*¹,*N*¹²-bis(ethyl)spermine (BE-3-4-3), which are not substrates for SSAT, may actually contribute significantly to the cytotoxic effects of these agents by removing normal cellular polyamines or by perturbing pools of acetyl-CoA and any other acetylase substrates [6–11]. The importance of SSAT in the response to polyamines and analogues is difficult to evaluate because no good inhibitors of its activity are available. Also, SSAT levels are very tightly regulated by polyamines at the levels of transcription, translation and protein degradation [2,12–14]. It is therefore very difficult to alter SSAT levels independently of alterations in polyamine content. Transfection with plasmids expressing SSAT from viral promoters leads to only small increases in SSAT activity due to the post-transcriptional repression, unless polyamines or analogues are also added [14,15]. More knowledge of SSAT structure, function and expression is important in assessing the importance of this enzyme.

Previous studies using inactivation of SSAT by butane-2,3-dione and other arginine-specific reagents have indicated that one or more arginine residues are important for SSAT activity and that inactivation was prevented by acetyl-CoA. This sug-

gested that these residues may be involved in binding this substrate [16]. We have therefore altered all eight of the arginine residues in SSAT individually to alanine residues and determined the effects on activity and the ability of acetyl-CoA to protect against digestion by trypsin. Such digestion has been shown to occur at a unique site sequence, namely Lys¹⁴¹-Arg¹⁴²-Arg¹⁴³ [17]. These results indicate that at least five of the arginine residues may be involved in SSAT activity and that three significantly affect acetyl-CoA binding.

We have also tested whether the active site of SSAT, which is a multimeric protein, is made up of residues contributed by separate monomers at the subunit interface, by co-expressing the inactive R101A (Arg¹⁰¹ → Ala) mutant with an E152K (Glu¹⁵² → Lys) mutant, previously shown to inactivate SSAT [17]. Such co-expression led to a restoration of activity. This led us to construct the double mutant R101A/E152K and test this as a dominant negative protein when co-expressed with wild-type SSAT. The results show that this mutant does act in this way both *in vitro* in a coupled transcription/translation system and in COS-7 cells transfected with a plasmid expressing the mutant SSAT from the simian-virus-40 late promoter

EXPERIMENTAL

Mutagenesis and plasmid construction

The plasmid pSAT9.3 containing the SSAT cDNA in the Bluescript vector [18] was used for site-directed mutagenesis as described in [17]. The sequence of the entire coding region of all mutant cDNAs was checked to ensure the absence of secondary mutations. The double mutant R101A/E152K was produced by

Abbreviations used: SSAT, spermidine/spermine-*N*¹-acetyltransferase; BE-3-4-3, *N*¹,*N*¹²-bis(ethyl)spermine; CAT, chloramphenicol acetyltransferase; R101A etc., Arg¹⁰¹ → Ala mutant etc.

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digesting each of the Bluescript plasmids containing the single mutants R101A and E152K [17] with restriction endonucleases *SphI* and *BamHI*. *SphI* cuts within the cDNA coding region located between the nucleotides coding for Arg¹⁰¹ and Glu¹⁵². The larger (3.5 kbp) fragment containing the R101A mutation and the smaller (288 bp) fragment containing the E152K mutation were then ligated together to form a Bluescript plasmid, p9.3-R101A/E152K.

Expression of wild-type and mutant SSATs *in vitro*

This was carried out using the T7 promoter of plasmid pSAT9.3 [18] in the TNT[®] coupled reticulocyte lysate system (Promega, Madison, WI, U.S.A.) as previously described [17] with the amount of plasmid indicated in the legends and a 12.5 μ l or 25 μ l assay volume. The extent of SSAT synthesis was determined by using [³⁵S]methionine in the translation assay, separating the products by SDS/PAGE and quantifying the amount of SSAT protein formed by densitometric scanning of the band corresponding to the 20 kDa SSAT protein subunit. The SSAT activity of the protein was measured by monitoring the production of [¹⁴C]acetylspermidine in an assay mixture which contained either 0.1 mM or 3 mM spermidine as indicated and 16 μ M [¹⁴C]acetyl-CoA (50 mCi/mmol) [17]. An aliquot from the translation assay was used as a source of SSAT. With the control SSAT plasmid, this aliquot represented only 0.5 μ l of the 12.5 μ l used for transcription/translation. For mutants with lower activities, larger aliquots were used to increase the sensitivity of the assay. When the apparent K_m for spermidine was determined, spermidine was varied from 0.05 mM to 3 mM. When the apparent K_m for [¹⁴C]acetyl-CoA was measured, the acetyl-CoA concentration was varied over the range 1–80 μ M.

Degradation of SSAT by trypsin

Trypsin digestion was carried out by using 125 ng of trypsin for 1 h at 30 °C in a total volume of 50 μ l of 50 mM Tris/HCl (pH 7.5)/2.5 mM dithiothreitol and 0.1 mM EDTA and 5 μ l from the TNT reaction containing the [³⁵S]SSAT, as described [17].

Expression of R101A/E152K mutant SSAT in COS-7 cells

The pEUKC1 plasmid previously used to express the control SSAT sequence [14] was used to express the R101A/E152K mutant SSAT. The p9.3-R101A/E152K plasmid was cut with *XhoI* and *BamHI*, and the 0.8 kbp fragment containing the entire SSAT coding region inserted into the pEUKC1 plasmid cut with the same enzymes [18]. This plasmid, pEUK-R101A/E152K, was then used to transfect COS-7 cells. The cells were cultured as previously described [14]. Aliquots were seeded at $(2.2\text{--}2.5) \times 10^5$ cells/60-mm-diameter dish and grown for 12 h. The cells were washed once in serum-free medium prior to transfection with 2–3 μ g of plasmid DNA using 20–30 μ g of lipofectamine (Gibco-BRL, Gaithersburg, MD, U.S.A.). The culture medium was replaced at 4–5 h after the start of transfection and the cells grown for a total period of 24–72 h as indicated in Table 3 (below). Treatment with 10 μ M BE-3-4-3 was started at 1 h prior to transfection, and the drug was present throughout the experiment.

RESULTS

Effect of mutations on SSAT activity

The effects of mutations on SSAT activity were determined by transcription/translation of the mutant or control cDNA in a

Table 1 Effect of mutations on SSAT activity

SSAT was synthesized in the TNT system using 0.125 μ g of plasmid per 12.5 μ l reaction as described in the Experimental section. The SSAT activity produced was measured in the standard assay with 3 mM spermidine and 16 μ M [¹⁴C]acetyl-CoA. The results are expressed as the percentage of the control activity. Mutants H49A, H53A, H63A, H69A, C14A, C54A and C120A had little effect on SSAT activity, which was 60% or more of control values in all cases (results not shown).

SSAT cDNA	SSAT activity (% of control)	Apparent K_m (μ M) for:	
		Spermidine	Acetyl-CoA
Control	100	70	3.6
R7A	6	21	6.0
R19A	38	130	3.5
R101A	3	80	$\approx 550.0^*$
R119A	100	63	3.8
R121A	80	51	3.6
R142A	55	55	25.0
R143A	11	95	68.0
R155A	23	690	4.7
K141S/R142A/R143S	< 1	ND [†]	ND [†]
H126A	12	1100	4.2
C122A	36	142	1.9
R101A/E152K	< 1	ND [†]	ND [†]

* This value could not be determined accurately because the maximal concentration of [¹⁴C]acetyl-CoA that could be used in the assay system was 80 μ M.

[†] ND, Not determined.

reticulocyte-lysate system monitored by measurement of the SSAT activity in the presence of 3 mM spermidine and 16 μ M acetyl-CoA. Previous studies have shown that mutants affecting SSAT activity are easily assayed in this way. It should be noted that, since the assay conditions include high substrate concentrations, even mutants in which substrate binding was greatly affected give readily measurable activities. An apparent K_m value for both spermidine and acetyl-CoA was obtained for all mutants. All of the eight arginine, five histidine and four cysteine residues in the SSAT protein, which consists of 171 amino acids [18], were investigated in this way by replacing them with alanine residues (Table 1).

None of the four cysteine residues were critical for SSAT activity. The greatest effect of altering these residues was that which occurred when Cys¹²² was replaced by Ala (C122A mutant), namely a reduction in activity, under the standard conditions, of 64%. However, all of the cysteine-to-alanine mutations reduced the accumulation of SSAT protein by 57–63%, suggesting that these amino acids may be important in the stability of the protein. Of the five histidine residues, only the mutation H126A produced a major decrease in SSAT activity, reducing it by 88%. This mutation led to a 15-fold increase in the apparent K_m for spermidine, suggesting a role for His¹²⁶ in binding the amine substrate.

Major effects in reducing SSAT activity were produced by changing any one of Arg⁷, Arg¹⁹, Arg¹⁰¹, Arg¹⁴³ or Arg¹⁵⁵ residues to Ala. Of these changes, only the R155A alteration increased the K_m for spermidine significantly (by about 10-fold). Mutations of Arg¹⁰¹ and Arg¹⁴³ increased the K_m for acetyl-CoA by about 150-fold and 20-fold respectively (Table 1). The mutation R142A had a smaller effect on the K_m for acetyl-CoA, increasing this value by 7-fold. When Arg residues at both position 142 and 143 were mutated in the mutant K141S/R142A/R143S, all activity was lost. These results suggest

Figure 1 Effect of acetyl-CoA on cleavage of SSAT and mutants by trypsin

(a) Shows the effects of increasing concentrations of acetyl-CoA on the degradation of control SSAT. (b) Shows the effects of 50 μ M acetyl-CoA on the degradation of control SSAT and mutants R101A, R155A and K141S/R142A/R143S.

that the arginine residues forming the acetyl-CoA binding site are those at positions 101 and 142/143.

Ability of substrates or analogues to protect SSAT from degradation by trypsin

Previous studies have shown that SSAT is readily degraded by trypsin digestion with primary cleavage occurring at the Lys¹⁴¹-Arg¹⁴²-Arg¹⁴³ site and that this degradation is completely blocked when BE-3-4-3 or other polyamine analogues are added [17]. As shown in Figure 1(a), addition of acetyl-CoA also prevents tryptic digestion of SSAT, with a maximal effect at 50 μ M. This protection was abolished by mutants R101A, R155A and K141S/R142A/R143S (Figure 1b) and by R142A and R143A (results not shown). This is consistent with the suggestion that these residues are involved in acetyl-CoA binding. It can be seen in Figure 1(b) that mutant R101A was more resistant than the control SSAT to trypsin cleavage. This was also true for mutant R142A (results not shown), but this may be because the mutation involves the primary cleavage site. The mutant

K141S/R142A/R143S lacks this primary site completely but, as previously reported [17], was cleaved by trypsin at Lys¹⁶¹ (Figure 1b).

Complementation of mutants

SSAT is a multimeric enzyme which appears to be a dimer when analysed by analytical ultracentrifugation (M. G. Fried and A. E. Pegg, unpublished work). This raises the possibility that the active site of the enzyme is made up at the dimer interface. In order to test this possibility, different inactive mutants were co-translated in order to examine whether heterodimers with restored activity could be obtained [19–23]. As shown in Table 2, the combination of the R101A mutant with mutant E152K, which has previously been shown to greatly reduce SSAT activity [17], did indeed produce far more activity than the expected sum when these mutants were translated separately. This complementation only occurred when the mutant cDNAs were co-translated. No increase was seen when the mutant monomers were synthesized separately and then combined, indicating that the subunits do not exchange freely (Table 2).

Dominant negative mutant of SSAT

The results described above, indicating that the active site of SSAT involves residues contributed by both subunits, suggested that a dominant negative mutant could be produced such that the combination with control SSAT would lead to the loss of activity of the native SSAT. Such a mutant was therefore constructed by combining the R101A and E152K mutations in a single cDNA. This R101A/E152K double mutant completely lacked SSAT activity (Table 1). Co-translation of this double mutant with pSAT9.3 producing the control SSAT did indeed lead to a substantial loss of activity (Figure 2a). This reduction in activity of the control SSAT was not due to the interference of the mutant plasmid with the synthesis of the control SSAT, since (i) the level of synthesis was proportional to the total amount of plasmid added (Figure 2b) and (ii) co-translation of the single R101A or E152K mutants (not shown) or the inactive K141S/R142A/R143S mutant, which would have a similar effect on synthesis, did not produce the same large reduction in SSAT activity (Figure 2a).

In order to test whether the R101A/E152K mutant SSAT was able to reduce SSAT in cells, the mutant cDNA was subcloned into the pEUK expression vector, and this plasmid was transfected into COS-7 cells in which SSAT activity was induced by the addition of BE-3-4-3. As previously described, such induction

Table 2 Effect of co-translation of SSAT mutants on SSAT activity

Plasmids expressing the SSAT control or mutants indicated were incubated in the TnT system for 45 min and 3 μ l aliquots of the mixture used for the assay of SSAT activity. These assays were carried out using 0.1 mM spermidine and 16 μ M [¹⁴C]acetyl-CoA and the SSAT activity is given as radioactivity (c.p.m.) incorporated into [¹⁴C]acetylspermidine. Unless indicated otherwise, where mutant cDNAs were mixed, they were co-translated.

SSAT cDNA	Amount of plasmid (μ g)	SSAT activity (c.p.m.)	Increase over sum (c.p.m.)
Control	0.0625	6926	—
Control	0.125	11 536*	—
R101A	0.0625	292	—
E152K	0.0625	299	—
R101A + E152K	0.0625 of each	4540	4031
R101A + E152K	0.0625 of each translated separately	648	139

* This value is slightly underestimated because the assay linearity declines when more than 10 000 c.p.m. are converted into the product.

Figure 2 Effect of co-translation of mutant SSAT plasmids on activity of SSAT derived from pSAT9.3

The Bluescript plasmids containing mutated SSAT cDNA sequences were translated in a total volume of 25 μ l containing 31.25 ng of the control pSAT9.3 plasmid and 31.25 (1:1), 62.5 (2:1), 94 (3:1), 125 (4:1), 156 (5:1), 187.5 (6:1) and 219 ng (7:1) of the plasmid expressing the mutant cDNA as indicated. (a) Shows the SSAT activity obtained after co-translation of pSAT9.3 with increasing amounts of either the plasmid coding for R101A/E152K (open circles and filled squares) or that coding for K141S/R142A/R143S (filled circles and closed squares). The experiment was carried out several times with identical results. The filled and open symbols represent two different experiments. (b) Shows the labelled SSAT protein produced in the assays combining wild-type and the R101A/E152K mutant and densitometric quantification of this band.

gives rise to a large increase in the endogenous SSAT activity of these cells [14]. Three separate experiments were carried out, and in all cases SSAT activity was strikingly reduced by pEUK-R101A/E152K compared with mock transfection or transfection with the plasmid pEUKCAT [which expresses CAT (chloramphenicol acetyltransferase)] (Table 3). Western blots (not shown) revealed that the total SSAT protein was increased greatly in the cultures transfected with pEUK-R101A/E152K, showing that the R101A/E152K mutant SSAT is stable in the cell. Despite the increase in total SSAT protein, SSAT activity was reduced by 50–80 % at 48 h after transfection, depending on the experiment. There was a 66 % reduction at 72 h after

Table 3 Effect of R101A/E152K mutant SSAT expression on SSAT activity in COS-7 cells treated with BE-3-4-3

COS-7 cells were treated with 10 μ M BE-3-4-3 and 1 h later transfected using lipofectamine with pEUK-CAT or pEUK-R101A/E152K or no plasmid as shown. SSAT activity was determined in the cultures at the time shown after transfection. The results shown are the means $\pm$ S.D. for four measurements for each experiment. The treatment with BE-3-4-3 increased the SSAT activity in the COS-7 cells from 0.2 ± 4.3 nmol/min per mg at 48 h.

Transfection	Time (h)	SSAT activity (nmol/min per mg)
Expt. 1		
pEUK-CAT	48	17.9 ± 2.2
pEUK-R101A/E152K	48	7.6 ± 0.6
pEUK-CAT	72	26.5 ± 4.3
pEUK-R101A/E152K	72	8.9 ± 0.6
Expt. 2		
pEUK-CAT	24	6.1 ± 0.2
pEUK-R101A/E152K	24	5.1 ± 0.1
pEUK-CAT	48	22.2 ± 1.1
pEUK-R101A/E152K	48	11.2 ± 0.5
Expt. 3		
None	24	6.5 ± 0.6
pEUK-R101A/E152K	24	3.7 ± 0.1
None	48	24.4 ± 4.3
pEUK-R101A/E152K	48	5.0 ± 0.1

transfection and a much smaller, 15 %, reduction at 24 h. The latter is expected, since plasmid-derived protein expression is quite low at this time. The variation from one experiment to another is likely to be due to the different transfection efficiencies.

DISCUSSION

The coupled transcription/translation system provides a very convenient means for rapidly assessing the effects of mutations on protein function with SSAT [17]. The endogenous SSAT activity in the reticulocyte lysate is negligible, and sufficient SSAT protein is produced to give a very high activity in the standard assay when control SSAT is used. Therefore, activity could be assayed accurately using only a small fraction of the total for the control SSAT, and activities of proteins with mutations that drastically affect activity could still be measured. Since the assays were carried out with crude extracts containing protein synthesized *de novo*, no attempt was made to express the activities as k_{cat} values. Instead, all were given as the percentage of the control activity in the standard assay system. The extent of SSAT synthesis was determined by densitometric scanning of the labelled protein band when [35 S]methionine was included in the translation system, and the results for the SSAT activity were corrected for the amount of protein present. However, these corrections were small and do not affect the results, since most of the mutations (except for the cysteine mutants described above) had little effect on the amount of SSAT protein that accumulated in the 45 min synthesis period.

The results show that five of the eight Arg residues are important in the activity of SSAT. However, it is likely that the inactivation of the protein by butane-2,3-dione, phenylglyoxal or camphorquinone-10-sulphonic acid, which was blocked in the presence of acetyl-CoA [16], is due to reaction with one or more of Arg¹⁰¹, Arg¹⁴² and Arg¹⁴³. Alteration of these residues changes the apparent K_m for acetyl-CoA and prevents protection from

trypsin by acetyl-CoA (Table 1 and Figure 1). A similar examination of the key Arg residues in choline acetyltransferase was carried out by Wu and Hersh [24], who changed all of the conserved Arg residues individually to Ala in the rat enzyme. They found that acetyl-CoA binding required Arg⁴⁵². The sequence context of this residue (-SASXRRFXXG-) has only marginal similarity to that for Arg¹⁰¹ (-MSDYRGFGIG-) or Arg¹⁴²Arg¹⁴³ (-NFYKRRGASD-) in SSAT.

Clarification of the roles of these Arg residues will require a knowledge of the three-dimensional structure of the SSAT protein. Crystals of human SSAT have been obtained and are being used for this purpose (S. E. Ealick, N. Campobasso and A. E. Pegg, unpublished work). Although the crystal structure of CAT is known, this does not provide a model for SSAT because the protein is a trimer, and the acetyl-CoA binding site does not involve interactions with arginine residues [25,26]. This contrasts with the acetyl-CoA binding site of citrate synthase in which three arginyl side chains co-ordinate the phosphoanions of CoA [27].

Although studies of protein structure and function by site-directed mutagenesis can be criticized on the grounds that the mutated protein may show gross changes in structure leading to a phenotype not directly related to the residue mutated, it is unlikely that our identification of Arg¹⁰¹ and Glu¹⁵² as key residues is compromised in this way. These proteins complement one another, showing that they still have the ability to form dimers and, when digested with trypsin, they show the characteristic cleavage to give a 16 kDa fragment rather than a more general fragmentation expected from a partially denatured protein. In fact, mutant R101A was more resistant to trypsin than the wild-type protein. Also, the R101A/E152K double mutant is still able to form dimers with the wild-type SSAT subunit, since it inactivates the protein and is accumulated in the cell, showing that it is stable.

The co-expression of two inactive mutant proteins to form a hybrid active protein, or the loss of activity when a wild-type protein is co-expressed with an inactive mutant, has been used in several studies to provide information on the contribution of subunit interfaces to ligand binding and catalysis [19–23,25]. Therefore, the restoration of activity when mutants R101A and E152K were co-expressed, and the reduction of wild-type SSAT activity when co-expressed with the double mutant R101A/E152K, provides convincing proof that the active form of SSAT is a multimer of the 20 kDa subunit and that the active site involves Arg¹⁰¹ from one subunit and Glu¹⁵² from another. Although previous studies have suggested that native SSAT from mammals may have an M_r of 60000–80000, suggesting that the structure could be a trimer or tetramer (reviewed in [2]), recent studies using analytical ultracentrifugation of the purified recombinant human SSAT show that the molecule behaves exactly as a dimer (M. G. Fried and A. E. Pegg, unpublished work). Some combinations of other mutants also gave rise to more activity than that expected from the results when each was expressed separately. In particular, the combination of E152K with R143A (or K141S/R142A/R143S) and the combination of R101 with M167Stop (i.e. Met¹⁶⁷ → stop codon) gave an increase in activity, whereas no increase was seen with R101A and R143A together or E152K and M167Stop together (C. S. Coleman and A. E. Pegg, unpublished work). This suggests that the same subunit contributes both Arg¹⁰¹ and Lys¹⁴¹-Arg¹⁴²-Arg¹⁴³ (which are

involved in acetyl-CoA binding) and the other contributes Glu¹⁵² and the carboxy end of the molecule, moieties that have been implicated in polyamine binding.

The transient transfection of COS-7 cells with pEUK-R101A/E152K provides convincing evidence that the R101A/E152K mutant SSAT can be expressed to adequate levels in the cell to be useful as a tool for the reduction of SSAT activity. Even with the lipofectamine protocol used, which is a relatively efficient means of transfecting COS-7 cells, only 50–80 % of the cells take up the added DNA. The residual SSAT activity is thus likely to be contributed by those cells that did not express the mutant SSAT. Therefore, the selection of clones of cells that stably express this mutant from a viral promoter should prevent the increase in SSAT associated with exposure to exogenous polyamines or analogues. This provides a valuable research tool for determining the importance of the induction of SSAT activity in the growth inhibition and anti-tumour effects produced by these agents.

This research was supported by grant GM-26290 to A.E.P. from the National Institutes of Health. C.S.C. was the grateful recipient of a Wellcome Trust Travel Grant.

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