Site of Pyruvate Formation and Processing of Mammalian S-Adenosylmethionine Decarboxylase Proenzyme*

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Mammalian S-adenosylmethionine decarboxylase was expressed at a high level in an Escherichia coli mutant deficient in this enzyme. The proenzyme form of this enzyme was cleaved and processed to the mature decarboxylase which contains two pairs of nonidentical subunits, the larger of which contains a pyruvate prosthetic group. In order to determine the site of formation of the pyruvate, two approaches were used. First, the mammalian S-adenosylmethionine decarboxylase produced in E. coli was purified to homogeneity and the pyruvate converted to alanine by a reductive amination. The large subunit was then isolated by reversed phase high pressure liquid chromatography and the amino-terminal sequence determined and compared with the sequence of the proenzyme derived from its cDNA. These results indicated that the bond between glutamic acid 67 and serine 68 was the site of cleavage. Second, each of the serine residues in portion of the proenzyme likely to contain the cleavage site were altered by site-directed mutagenesis and the RNA produced from plasmids containing these mutations was translated in a reticulocyte lysate. The translation products were tested for processing and for S-adenosylmethionine decarboxylase activity. Altering the serine residues at positions 50, 66, and 69 to alanines had little effect but changing serine at position 68 to alanine completely prevented both processing and activity. These results indicate that the serine residue at position 68 of the proenzyme which is in the underlined position in the sequence -Leu-Ser-Glu-Ser-Ser-Met- is the residue which is converted to the pyruvate prosthetic group in human S-adenosylmethionine decarboxylase.

S-Adenosylmethionine (AdoMet) decarboxylase is a key enzyme in the biosynthesis of polyamines (1-3). The mammalian enzyme is known to be activated by putrescine and to contain a pyruvate prosthetic group (4-7), but detailed studies of the enzyme structure, mechanism of action and the origin of the pyruvate group have been hampered by the small amount of enzyme protein present in even the richest mammalian sources. Recently, we have reported the isolation and sequence of cDNA clones for this enzyme (8). These clones contained an open reading frame corresponding to a polypeptide of about M, 35,000. Translation of mRNA isolated from rat prostate and of the active enzyme from these cDNAs in a reticulocyte lysate indicated that the enzyme is synthesized as a proenzyme of this size which is then processed in a putrescine-stimulated reaction which generates two subunits of approximately M, 32,000 and 6,000 (8-11). This processing presumably generated the pyruvate prosthetic group which is attached to the larger subunit (12). The precursor of the pyruvate is likely to be an internal serine residue as has been described for a number of bacterial enzymes containing this cofactor (13-16). Although the probable amino acid sequence of the AdoMet decarboxylase proenzyme has been derived from the cDNA sequence (8), direct analysis has not been possible since this enzyme is a very minor cellular component and only a very small percentage of it is present as the nonprocessed form. In order to obtain sufficient material for investigation of the processing reaction and formation of the pyruvate we have expressed the protein in E. coli. Also, site-directed mutagenesis of the serine residues which might serve as the precursor of the pyruvate has been carried out and the resulting mutants tested for their ability to undergo processing and generate the active enzyme. The results of the two approaches both indicate that the serine residue at position 68 of the proenzyme is the source of the pyruvate.

EXPERIMENTAL PROCEDURES

Materials—The pCQV2 expression vector originally constructed by Dr. C. Queen (17) was kindly provided by Dr. P. Coffino, Department of Microbiology, University of California San Francisco. E. coli strain HT551 was generously provided by Drs. H. and C. W. Tabor, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. MGBG-Sepharose was prepared as described previously (18). Antiserum to homogeneous rat prostate AdoMet decarboxylase was raised in rabbits (17). AdoMet-[14COOH] and [35S]methionine were purchased from Du Pont-New England Nuclear. Biochemical reagents were purchased from Sigma, Pharmacia LKB Biotechnology Inc., Bio-Rad, and Bethesda Research Laboratories. RNasin and pGEM3Zf(-) were obtained from Promega. The sequences coding for the human AdoMet decarboxylase were isolated from pSAM1h (8).

Site-directed Mutagenesis of Residues in Human AdoMet Decarboxylase—A PvuII-Xbal fragment from the human AdoMet decarboxylase cDNA (8) was isolated, gel-purified, and subcloned into pGEM3Zf(-) using the Smal and Xbal sites to produce pCM9. This plasmid contains 1150 bp from the human cDNA including 94 bp from the 5'-noncoding region, the complete 1002 bp from the coding sequence and 63 bp from the 3'-noncoding region. The sequence of the insert corresponding to the coding region was checked and cor-

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responded to the published sequence (8) except that base 437 was C, base 889 was C, and base 890 was G (numbering from the A of the initiation codon as 1). These changes alter the derived amino acid sequence from glycine at position 146 to alanine and the alanine at 297 to arginine.

Product-directed mutagenesis was carried out by a modification of the method described by Kunkel (19). Single-stranded DNA containing uracil was prepared from pCM9 grown in E. coli strain CJ236 in the presence of M13K7 helper phage and purified on 3% NuSieve GTG agarose gels (FMC Products, Rockland, ME). The orientation of the 5' origin of replication in the pCM9 was such that the single-stranded DNA produced contained the DNA sequence equivalent to sense mRNA, so the following antisense oligodeoxynucleotides were synthesized for use as mutagenic primers to produce the indicated amino acid changes (mismatches are underlined): 5'-GT-CAC-TAT-GAT-TG-GC-AGTTG-3'; Ser-50-Ala, 5'-CT-CAT-TAT-GAT-TG-GC-AGTTG-3'; Ser-66-Ala, 5'-AC-CA-ACAT-GAAT-ATCTCAC-3'; Ser-68-Asp, 5'-GAC-AAACAT-GCTAGCCTCA-3'; Ser-79-Ala, 5'-CA-CAACAT-GAAT-ATCTCAC-3'; and Ser-80-Ala, 5'-GAGAACAAT-GCTAGCCTCA-3'. The mutagenic primers were phosphorylated, annealed to the uracil containing single-stranded DNA in a ratio of 25:1 (primertemplate) and a complementary strand synthesized using T4 DNA polymerase (19) or Sequazyme (20). The double-stranded DNA formed was introduced into E. coli DH5m MCR cells by electroporation (21).

Amplification-resistant colonies were picked, grown up overnight, and used for isolation of small amounts of DNA (22). This DNA was then sequenced (23) using a suitable primer to allow the putative mutation to be identified. Large scale preparations of DNA from the desired mutants were obtained from 500-ml cultures using the alkaline lysis method (24) followed by centrifugation in CsCl.

Transcription and Translation of mRNA for AdoMet Decarboxylase—Aliquots of 50 µg of purified DNA from pCM9 and mutants derived from it were linearized at a site 63 nucleotides 3' from the coding region by cutting with XbaI. In in vitro transcription was carried out using T7 RNA polymerase. A total volume of 45 µl containing 10 µg of linearized DNA, 10 µl of T7 RNA polymerase buffer, 5 mM dithiothreitol, 2 mM ATP, 2 mM UTP, 2 mM CTP, 0.4 mM GTP, 2 mM MgCl2, and 50 units RNAase was preheated for 5 min at 37 °C, and then 120 mM potassiumacetate, 1 mM magnesiumacetate, 50 µM each amino acid except methionine, 1.5 mM [35S]methionine, 25 units of RNasin, 200 µM putrescine, and 0.25 µg of ribonuclease-free deoxyribonuclease I was added. The mixture was incubated further for 15 min at 37 °C and then EDTA was added to 45 mM, and the solution extracted with phenol and then with chloroform. The aqueous phase was then precipitated overnight with ammonium acetate/ethanol. RNA was pelleted by centrifugation, washed with 70% ethanol, and dried down in vacuo.

Prior to in vitro translation, the RNA was heated for 10 min at 65 °C and then cooled on ice. Translation of the RNA to generate labeled product used to study the processing reaction was carried out in a reaction medium containing 0.5% rabbit reticulocyte lysate, 1 mM magnesium acetate, 1 mM dithiothreitol, 0.5% rabbit reticulocyte lysate. After incubation for 90 min at 30 °C, 3 µg of ribonuclease A was added to the reactions, and the incubation was continued for 15 min at 30 °C.

The carboxymethylated protein was purified by HPLC on a Hi-Pore 297 to arginine.

RESULTS

In order to obtain high-level expression of human AdoMet decarboxylase in E. coli, a part of the CDNA containing the coding sequence of human AdoMet decarboxylase was inserted into the expression vector pCQV2 (17). These constructs contain a bacteriophage λ promoter from which the AdoMet decarboxylase mRNA is transcribed and a thermolabile λ repressor so that transcription is prevented when the cells are cultured at 32 °C and is maximal when the temperature is raised to 42 °C (13). E. coli JM109 cells with and
without pCQV2A were grown at 32 °C to mid-log phase, an aliquot was transferred to 42 °C, and after a further 1 h, samples were collected from induced and noninduced cultures to determine AdoMet decarboxylase activity (Table I). The control cells did not contain any measurable AdoMet decarboxylase activity in the presence of 3 mM putrescine which activates mammalian but not bacterial AdoMet decarboxylase. Cells containing pCQV2A showed some AdoMet decarboxylase activity at 32 and 42 °C this activity was increased about 50-fold. AdoMet decarboxylase activity was detected in extracts from cells both with and without the construct when assayed in the presence of 10 mM Mg²⁺, an activator of E. coli AdoMet decarboxylase. Thus, the E. coli AdoMet decarboxylase in these cells may interfere with the purification of the human protein.

To circumvent this problem, the mutant strain HT551, which lacks AdoMet decarboxylase and spermidine synthase (27), was transformed with pCQV2A or with the pCQV2 vector without insert. As seen from Table I, cells with only pCQV2 showed very little, if any, AdoMet decarboxylase activity 1 h after induction. The same was true for cells containing pCQV2A when grown at 32 °C, whereas at 42 °C the activity was almost as high as in JM109 cells carrying the same construct. The AdoMet decarboxylase activity expressed in crude extracts of HT551 cells was not reduced much when the assay was carried out in the absence of putrescine, but this may be explained by the fact that HT551 cells have a high intracellular putrescine level. After extracts had been precipitated with 80% saturated ammonium sulfate and then dialyzed against the assay buffer, the AdoMet decarboxylase activity was stimulated approximately 2-fold by the addition of putrescine but not by Mg²⁺ (results not shown). Further proof that the mammalian enzyme was being expressed was obtained by performing the assay in the presence of MGBG, a powerful inhibitor of mammalian AdoMet decarboxylase. This showed an IC₅₀ of approximately 1 μM for the enzyme, in good agreement with the reported value for rat AdoMet decarboxylase assayed under the same conditions (31).

The time course of AdoMet decarboxylase activity after induction in HT551 cells is shown in Fig. 2. The cells were grown to mid-log phase at the nonpermissive temperature (32 °C), a portion of the cell suspension was then transferred to the permissive temperature (42 °C) and maintained at this temperature for 1–24 h. Alternatively, the cells were shifted to 42 °C for 30 min and then transferred back to 37 °C. Samples were taken at different time points to determine AdoMet decarboxylase activity. Maximum activity was reached after 2–4 h at 42 °C. Longer incubations resulted in a substantial loss of AdoMet decarboxylase activity. This may be due to the deleterious effects of maintaining the bacteria at the higher temperature although the AdoMet decarboxylase activity also declined during prolonged incubation at 37 °C of cells which were induced to produce the enzyme by a transient period of 30 min at 42 °C to inactivate the λ repressor (Fig. 2).

To determine the rate of AdoMet decarboxylase synthesis and investigate the processing of the proenzyme, cells harboring the pCQV2A construct or pCQV2 only were grown in M9 minimal medium at the permissive and nonpermissive temperatures and labeled for 10 min with [³⁵S]methionine. Cells were sonicated, centrifuged, and the supernatant extracts were incubated with a mono-specific antibody to rat AdoMet decarboxylase (26) and then with protein A-Sepharose. Immunoprecipitated proteins were analyzed by SDS-PAGE (Fig. 3). Three major proteins were immunoprecipitated from the cells carrying the construct. One band having an apparent Mr of approximately 38,000 corresponded to the precursor of human AdoMet decarboxylase, and there was a less intense band of Mr 32,000 corresponding to the active enzyme subunit. There was also another labeled protein with an Mr of approximately 24,000 which reacted with the antisemur. This may be due to premature termination, internal initiation, or degradation. In order to study the processing of the proenzyme, aliquots of the labeled cells were treated with chloramphenicol to stop protein synthesis and incubated for a further 30, 60,
or 120 min (Fig. 3). Under these conditions, the $M_\text{r}$ 38,000 band disappeared and the subunit band of $M_\text{r}$ 32,000 increased indicating that processing of the human proenzyme occurs readily in E. coli. The $M_\text{r}$ 24,000 polypeptide did not seem to be processed in the same way as the proenzyme but it seemed much more unstable than the $M_\text{r}$ 32,000 subunit (Fig. 3). Chase reactions with unlabeled methionine or incubation for longer periods in the presence of $[^35]S\text{-labeled decarboxylated } S\text{-adenosylmethionine}$ in the presence of sodium cyanoborohydride as described previously (12). This permitted the labeling of the subunit AdoMet decarboxylase in the presence of $[^35]S\text{-labeled decarboxylated } S\text{-adenosylmethionine}$ in the presence of sodium cyanoborohydride as described previously (12). This procedure leads to the covalent attachment of the labeled decarboxylated AdoMet at the active site of the enzyme (12). As seen from Fig. 3, only the $M_\text{r}$ 32,000 protein was labeled, also indicating that the smaller protein does not represent an active enzyme.

Human AdoMet decarboxylase expressed in E. coli was purified from the bacteria in a similar four-step procedure involving streptomycin precipitation to remove nucleic acids, ammonium sulfate precipitation, affinity chromatography on MGBG-Sepharose, and fast protein liquid chromatography on Mono-Q (Table II). The resulting preparation had a specific activity of about 2 $\mu$mol of $CO_2$ produced per min/mg of protein and 140 $\mu$g was obtained from a 250-ml culture. This preparation appeared to be free of contaminating proteins when examined by polyacrylamide gel electrophoresis under denaturing conditions. The only proteins detected in the purified enzyme were the $M_\text{r}$ 32,000 band and a smaller diffuse band which ran near the gel front and corresponds to the smaller subunit of the AdoMet decarboxylase (Fig. 4). The activity of the purified enzyme was not affected by Mg$^{2+}$ but was stimulated 2.8-fold by the addition of 2.5 mM putrescine. Human AdoMet decarboxylase from HT29 colon tumor cells was stimulated to the same extent by putrescine under the same conditions. This stimulation is less than that found for the AdoMet decarboxylase from rat prostate which was stimulated about 7-fold by putrescine (results not shown) in agreement with previous observations (4, 18).

The purified human AdoMet decarboxylase was subjected to reductive amination to convert the pyruvate prosthetic group to alanine (28). As shown in Fig. 5, the treatment with ammonium acetate and NaCNBH$_3$ led to a rapid loss of enzyme activity which was much faster than the loss of activity during incubation in the same buffer lacking NaCNBH$_3$. The protein was then carboxymethylated and subjected to reversed phase HPLC. The fractions corresponding to large subunit were taken and subjected to protein sequencing using an Applied Biosystems gas phase sequencer. No readable sequence was obtained from the aliquot of the protein which had not been treated with NaCNBH$_3$ showing that the amino terminus of this subunit is blocked. The sample which had been treated with NaCNBH$_3$ gave the sequence Ala-Ser-Met-Phe-Val-Ser-Lys-Arg-Phe-Ile-Leu-X-Thr-Cys-Gly-. This corresponds exactly to the sequence from residues 68–83 of the proenzyme as derived from the cDNA (8) with the exception that residue 68 of the proenzyme is serine and indicates that the serine residue at position 68 is likely to be the source of the pyruvyl prosthetic group.

In order to obtain more information on the processing reaction, site-directed mutagenesis was carried out to change the serine residues which are present in the AdoMet decarboxylase proenzyme in a region which could on cleavage give rise to products of the correct size for the two subunits. The human cDNA was subcloned into the pGEM3Zf(−) vector, since this plasmid could be used both for the production of single-stranded DNA needed for the mutagenesis and for the synthesis of mRNA corresponding to the cDNA insert using T7 RNA polymerase. RNA was synthesized from the control and the mutant plasmids and was translated in a reticulocyte lysate in the presence of $[^35]S\text{-methionine}$. The labeled proteins formed were then separated by SDS-PAGE (Fig. 6) and the extent of conversion of the proenzyme to the $M_\text{r}$ 32,000 subunit determined (Table III). The translation was also carried out in the presence of unlabeled amino acids, and the enzymatic activity of the AdoMet decarboxylase synthesized was determined (Table III). As shown in Fig. 6 and Table III, the double change of Ser-68-Ser-69 to Ile-Ile and the single

![Fig. 3. Immunoprecipitation of AdoMet decarboxylase from HT551 cells carrying pQV2A.](image)

Table II

| Fraction                  | Total protein | Total units | Specific activity | Purification | Yield % |
|---------------------------|---------------|-------------|------------------|--------------|--------|
| Supernatant               | 39.2          | 540.0       | 13.8             | 1            | 100    |
| (NH$_4$)$_2$SO$_4$ precipitate | 21.5         | 521.9       | 24.3             | 2            | 97     |
| MGBG-Sepharose eluate    | 0.7           | 366         | 544.0            | 39           | 68     |
| Mono Q eluate            | 0.14          | 290         | 2025             | 147          | 54     |

*1 unit of enzyme activity is defined as releasing 1 nmol of CO$_2$/min.
change of Ser-68 to Ala completely prevented the conversion of the proenzyme to the subunit. However, the single changes of Ser-50 to Ala, Ser-66 to Ala, and Ser-69 to Ala had little if any effect on the conversion. The changes which blocked the conversion also completely abolished the enzyme activity, whereas the other mutations of the serines at positions 50, 66, and 69 had no effects on activity (Table III). These results are also consistent with the cleavage site of the proenzyme being between glutamic acid at position 67 and serine at position 68 with the pyruvate being formed from this serine.

**DISCUSSION**

The expression of human AdoMet decarboxylase in E. coli using the vector pCQV2A provides a convenient method to obtain large amounts of the mammalian protein which should be valuable for studies of the enzyme structure and mechanism of action. Although the protein expression was only at a level of 0.5–1% of the total cellular protein, the purification on MDBG-Sepharose and Mono-Q provides a rapid method to obtain the purified protein in high yield. The reason for the relatively low level of expression compared to other cDNAs expressed in this vector (17) is not clear, but it may be due to the instability of the human AdoMet decarboxylase in the bacteria (Figs. 2 and 3). The construction of pCQV2A led to two changes in the amino acid sequence of the encoded AdoMet decarboxylase substituting Asp-Pro for Glu-Ala at positions 2 and 6, and 5, Ser-68 to Ala, and Ser-69 to Ala. Full details of the reaction conditions and sample preparation and analysis are given under “Experimental Procedures.” The band seen in all lanes having M, of about 46,000 was found when no mRNA was added to the labeling of a protein present in the reticulocyte lysates by reaction with a degradation product of the [35S]methionine. Migration of protein standards of the M, indicated (X10^3) is shown on the left.

There is much interest in the possible use of inhibitors of polyamine biosynthesis for therapeutic purposes (33, 34). Although some potent inhibitors of AdoMet decarboxylase have been synthesized and these have a marked effect on cell growth, these compounds have a number of disadvantages, including in some cases a lack of stability and in others a lack of specificity (31, 33–36). The rapid turnover of AdoMet decarboxylase is also a problem in maintaining a complete inhibition of this step even when irreversible inhibitors are used. The conversion of the AdoMet decarboxylase proenzyme to the final pyruvoyl-containing enzyme is an attractive step for the design of specific inhibitors which would prevent the synthesis of spermidine and spermine.

Our experiments indicate that the pyruvate moiety of hu-

**FIG. 4. SDS-PAGE of the different steps of AdoMet decarboxylase purification.** AdoMet decarboxylase was purified from E. coli as described in the text. Aliquots from each step were analyzed on a 12.5% SDS-PAGE gel. Lane 1, crude extract after removal of nucleic acids by streptomycin sulfate (0.1 unit of AdoMet decarboxylase); lane 2, proteins precipitating between 35 and 65% ammonium sulfate (0.1 unit of AdoMet decarboxylase); lane 3, eluate from MGBG-Sepharose affinity column (3 units of AdoMet decarboxylase); lane 4, AdoMet decarboxylase purified on Mono-Q (3 units of AdoMet decarboxylase). Migration of protein standards of the indicated M, (X10^3) is shown on the right. A unit of enzyme activity is defined as releasing 1 nmol of CO_2/min.

**FIG. 5. Time course of AdoMet decarboxylase inactivation by reductive amination.** Purified AdoMet decarboxylase was incubated in 2 mM ammonium acetate, pH 6.5, 2.5 mM putrescine, and 0.1% Triton X-100 in the presence (C) or absence (O) of 100 mM NaCNBH_3. Samples were taken at the times shown and the AdoMet decarboxylase activity determined.
human AdoMet decarboxylase is derived from the serine residue at position 68. In all of the known enzymes which contain pyruvate in an amide linkage, this pyruvate is derived from an internal serine residue as first demonstrated by Snell (13, 14) for histidine decarboxylase from Lactobacillus 30a. There is virtually no similarity between the sequence surrounding the serine residue which becomes the pyruvate in the E. coli AdoMet decarboxylase proenzyme (-HLDKSHICV-) (29, 37) and the mammalian sequence (-VLSESSMFV-) and there is no similarity with the pyruvate precursor sequence of other pyruvyl enzymes, histidine decarboxylase (-TTASSFTGV-) (14, 28), and phosphatidyleserine decarboxylase (FKLGSVT-VIN-) (16). However, recent work in the Tabors' laboratory has shown that the yeast AdoMet decarboxylase proenzyme is converted to pyruvate at the sequence -LSESSLFV- (38) which corresponds closely to the mammalian sequence. It is probable that the conversion is a self-catalyzed reaction which involves an intramolecular reaction from the proenzyme and that the tertiary structure of the proenzyme is a critical feature in providing the correct environment for this conversion. The stimulation of the rate of processing of the mammalian AdoMet decarboxylase in the presence of putrescine (10) can then be explained by the change in the structure brought about by binding putrescine.

The large subunit of human AdoMet decarboxylase contains 266 amino acid residues, and the pyruvyl group at the amino terminus and has M, of 30,719, which is in reasonable agreement with the value of 32,000 obtained by SDS-PAGE (8, 12, 26). Assuming that the smaller subunit derived from the amino-terminal portion of the proenzyme contains all of the amino acid residues preceding the cleavage site, it consists of 67 amino acids and has M, of 7,681. This is also in reasonable agreement with the value of 6,000 obtained using Tricine-SDS-PAGE analysis of the labeled mRNA translation products (8). This subunit was not seen in previous preparations of the mammalian AdoMet decarboxylase presumably because of its small size and the limited amount of material available for analysis. However, it is clearly visible on SDS-PAGE analysis of the recombinant human enzyme (Fig. 4).

The system used for the production of mutations in the AdoMet decarboxylase and the analysis of the effects of these mutations on the processing of the proenzyme and the enzymatic activity has several advantages which can be used to study other aspects of these properties such as the putrescine activation and the active site. The mutations can easily be produced in the pCM9 vector and analyzed by synthesis of RNA from the T7 promoter followed by translation in the reticulocyte lysate as described here. The sequence containing any mutations of particular interest can then be excised from the pCM9 using Csp45I and SstI and inserted into the pCQV2A vector cut with the same enzymes. Using this vector, the mutated AdoMet decarboxylase can then be produced in large amounts in E. coli HT551.

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