Role of Cysteine-82 in the Catalytic Mechanism of Human *S*-Adenosylmethionine Decarboxylase†

Haishan Xiong, Bruce A. Stanley, and Anthony E. Pegg*

Department of Cellular and Molecular Physiology, The Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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ABSTRACT: *S*-Adenosylmethionine decarboxylase is a pyruvate-dependent enzyme. The enzyme forms a Schiff base with substrate, *S*-adenosylmethionine, through the pyruvoyl moiety. This facilitates the release of CO_2 from the substrate, which must then be protonated on the α carbon in order to permit hydrolysis of the Schiff base to release the product. The catalytic mechanism of human *S*-adenosylmethionine decarboxylase was investigated via mutagenic and kinetic approaches. The results of enzyme kinetic studies indicated that Cys-82 is a crucial residue for activity and this residue has a basic pK_a. Iodoacetic acid inhibited wild-type enzyme activity in a time- and pH-dependent manner but did not affect the already reduced activity of mutant C82A. Reaction of this mutant with iodoacetic acid led to approximately one less mole of reagent being incorporated per mole of enzyme $\alpha\beta$ dimer than with wild-type *S*-adenosylmethionine decarboxylase. Both wild-type and C82A mutant *S*-adenosylmethionine decarboxylases were inactivated by substrate-mediated transamination, but this reaction occurred much more frequently with C82A than with wild-type enzyme. A major proportion of the recombinant C82A mutant protein was in the transaminated form in which the pyruvoyl cofactor is converted into alanine. This suggests that incorrect protonation of the pyruvate, rather than the substrate, occurs much more readily when Cys-82 is altered. On the basis of these results, it was postulated that residue Cys-82 may be the proton donor of the decarboxylation reaction catalyzed by *S*-adenosylmethionine decarboxylase.

S-adenosylmethionine decarboxylase (AdoMetDC)[1] is an essential enzyme for the biosynthesis of polyamines and is one of a small class of decarboxylases that use a covalently bound pyruvate as a prosthetic group (*1*, *2*). These pyruvoyl-dependent decarboxylases form amines such as histamine, decarboxylated *S*-adenosylmethionine, phosphatidylethanolamine (a component of membrane phospholipids), and β-alanine (a precursor of coenzyme A), which are all of critical importance in cellular physiology and provide important targets for drug design. All known AdoMetDCs are synthesized in proenzyme forms and a cleavage at an internal Ser residue is required to activate the proenzyme (*3*, *4*). The cleavage site of human AdoMetDC is between Glu-67 and Ser-68 (underlined) in the motif -VLSESS- (*5*). The Ser-68 is converted to a pyruvoyl group during the proenzyme processing. Processing of the proenzyme generates two nonidentical subunits, termed α and β, which are both indispensable components of the mature enzyme (*5*−*7*).

The first step of AdoMetDC catalysis is the binding of *S*-adenosylmethionine (AdoMet) through the pyruvate prosthetic group that reacts to form a Schiff base adduct with the substrate. The pyruvoyl cofactor in this Schiff base linkage then acts as an electron sink to aid in the breaking of the C−COO^- bond and the elimination of CO_2 from the enzyme−substrate complex (*1*). To release the final product, decarboxylated *S*-adenosylmethionine (dcAdoMet), a protonation must occur at the α carbon of the product (reaction 3 in the upper pathway in Figure 1). Through this protonation, the pyruvate is regenerated and is available for another cycle of catalysis.

Very little detail is available concerning the AdoMetDC reaction. By using tritiated water in enzymatic assays, a primary tritium isotope effect of 4.5 was observed (*8*). This high isotope effect indicates a significant proton exchange of an active-site residue with solvent. This residue is very likely to be the proton donor discussed above (also see Figure 1). Identification of this residue should further the understanding of the catalytic mechanism of AdoMetDC and the design of inhibitors of this enzyme.

AdoMetDC cDNA sequences have been cloned from a wide variety of species (*9*−*11*). All of the known sequences except that from *Escherichia coli* show a significant degree of similarity with about 50 fully conserved residues in the core region of 300 amino acids. Although the *E. coli* enzyme shares very little primary sequence homology with the mammalian enzymes, almost all inhibitors of *E. coli* AdoMetDC, regardless of their inhibitory mechanisms, inhibit all

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* To whom correspondence should be addressed at the Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, 500 University Dr., Hershey, PA 17033. Phone 717-531-8564; Fax 717-531-5157; E-mail aep1@psu.edu.

[1] Abbreviations: AdoMet, *S*-adenosylmethionine; dcAdoMet, decarboxylated *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; DTT, dithiothreitol; IAA, iodoacetic acid; PVDF, poly(vinylidene difluoride); PCR, polymerase chain reaction; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TCA, trichloroacetic acid.

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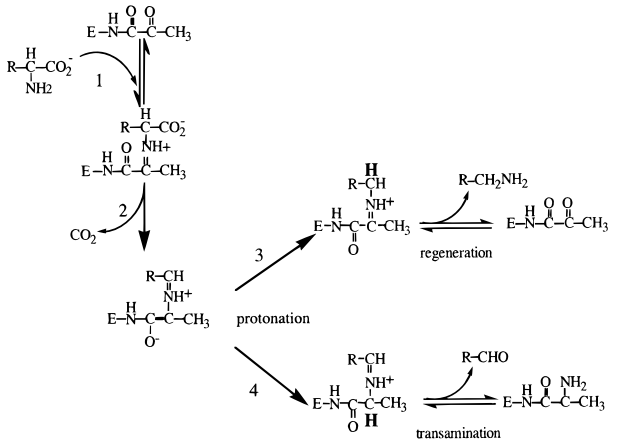

FIGURE 1: Catalytic mechanism of AdoMetDC and the substrate-mediated inactivation. The AdoMetDC enzyme is represented as E with the pyruvate prosthetic group at the N-terminus. The α-carbon, carboxylate, and primary amine groups of the substrate AdoMet are shown in full with the rest of the molecule represented by R. The proton that is transferred on the enzyme−product complex during protonation is shown in boldface type.

other AdoMetDC enzymes [see review (*3*)]. The lack of inhibitor specificity suggests that all the AdoMetDC enzymes utilize the same catalytic mechanism and that they have structurally similar active sites.

All known pyruvoyl enzymes can be inactivated by a substrate-mediated transamination in which AdoMetDC undergoes an incorrect protonation during enzymatic reaction (Figure 1, reaction 4 in the lower pathway). This protonation occurs on the pyruvate instead of the α-carbon of the product. A product in aldehyde form is released and the pyruvate prosthetic group is converted to an alanyl moiety. This transamination of the covalently bound pyruvate prosthetic group irreversibly inactivates the enzyme (*1*, *2*, *12*).

There is only one short sequence (-STCGVISPLKAL-) in the *E. coli* AdoMetDC (*13*) that shows a strong similarity to an equivalent region (-−KTCGTTLLLKAL-) in the mammalian AdoMetDCs. This region contains a cysteine residue (Cys-140) that is equivalent to Cys-82 in the human enzyme. All other known AdoMetDCs also have a cysteine in the equivalent position. Site-directed mutation of human Cys-82 to Ala showed that the protein lost all measurable (>95%) activity when assays were conducted in a coupled transcription/translation reticulocyte lysate system (*14*). Additional evidence that this cysteine residue may be involved in the active site of AdoMetDC was obtained in studies of the substrate-mediated inactivation of *E. coli* AdoMetDC (*15*). It was found that the substrate-mediated transamination of *E. coli* AdoMetDC results in a covalent modification of residue Cys-140 by an acrolein-like molecule derived from the aldehyde derivative of the substrate AdoMet (*16*).

We have therefore compared the properties of the wild-type and the C82A mutant human AdoMetDC in detail. These studies indicate that Cys-82 is extremely important in the catalytic mechanism of AdoMetDC and are consistent with the hypothesis that this residue acts as the proton donor.

MATERIAL AND METHODS

Materials. The pQE plasmids and the mouse anti-His-tag monoclonal antibody were obtained from Qiagen (Chatsworth, CA), and the Talon metal affinity resin was purchased from Clontech. Rabbit anti-human AdoMetDC antiserum was generated by immunizing rabbit with purified recombinant human AdoMetDC by the method previously described for AdoMetDC purified from rat prostate (*17*). *E. coli* competent cells were purchased from Stratagene (La Jolla, CA). The restriction enzymes used were from Gibco BRL (Gaithersburg, MD), Promega, and New England Biolabs (Beverly, MA). Iodoacetic acid (IAA) was obtained from Sigma Chemical Co (St. Louis, MO). Poly(vinylidene difluoride) (PVDF) membranes were purchased from Millipore (Bedford, MA). [^{14}COOH]AdoMet and iodo[2-^{14}C]-acetic acid were from Amersham Life Science, Inc., Arlington Heights, IL. Unlabeled AdoMet was purchased from Aldrich (Milwaukee, WI). All other chemicals were from Fisher (Pittsburgh, PA). The [^{35}S]dcAdoMet was prepared enzymatically as previously described (*18*).

Plasmid Construction. The human AdoMetDC cDNA was subcloned from pCM9 (*5*) into a pQE30 plasmid, which produces a protein with a $(His)_6$ tag to facilitate protein purification. The polymerase chain reaction (PCR) was carried out with the pCM9 plasmid as template, using Vent DNA polymerase (New England BioLabs). The two primers used were 5′-gctagtctcacgggatccgaagctgc-3′ and 5′-atttaggtgacactatag-3′. The 1.1 kb PCR product was digested with *Bam*HI and *Sal*I and was ligated into the pQE30 vector digested with the same enzymes to form pHIS-hSAM. This construct replaces the first two amino acids of human AdoMetDC (ME-) with MRGS$(H)_6$GSE-. The mutation C82A was generated in the pCM9 vector previously (*14*). To insert the C82A mutation into pHIS-hSAM, the pCM9-C82A plasmid was digested with *Csp*45I and *Sal*I. The 1 kb digestion product was then ligated into the pHIS-hSAM vector cut with the same enzymes. The resulting plasmid was called pHIS-hSAMC82A. The coding regions of all of the above plasmids were sequenced to ensure that only the desired mutations were present.

Purification of His-Tagged Human AdoMetDC Proteins. Plasmids pHIS-hSAM and pHIS-hSAMC82A were transformed into XL1-Blue *E. coli*. After selection on plates containing ampicillin, cells were grown at 37 °C to a density corresponding to A_{600} of 0.6 and expression of AdoMetDC was then induced by the addition of IPTG to a final concentration of 0.3 mM. After 3 h, the cells were harvested and washed once with buffer X (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2.5 mM putrescine, and 0.1 mM phenylmethanesulfonyl fluoride) at 4 °C. The cell pellet was then resuspended in buffer X and homogenized with a French press. The suspension was centrifuged at 20000*g* for 30 min and the supernatant was taken and applied to a 1 mL column of Talon resin preequilibrated with buffer X. The column was washed with buffer X containing 10 mM imidazole and the protein was eluted with buffer X containing 200 mM imidazole. The eluted protein was immediately passed through a Sephadex G-25 column preequilibrated with buffer Y [10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol (DTT), 2.5 mM putrescine, and 0.1 mM phenylmethanesulfonyl fluoride] to remove the imidazole, and the protein was stored at −80 °C until use.

Western Blotting Analysis. Purified human AdoMetDC proteins were resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) on a 12.5% gel. Proteins were electrophoretically transferred

onto PVDF membrane in transfer buffer (10 mM Caps, pH 11, and 20% methanol). Western blotting analysis was performed with the Western-light Plus kit (Tropix, Bedford, MA) following the manufacturer's recommendations.

Standard AdoMetDC Activity Assay. AdoMetDC activity was assayed by measuring the production of $^{14}CO_2$ from [^{14}COOH]AdoMet. The assay was carried out essentially as described (*19*) using hyamine hydroxide to trap the $^{14}CO_2$, which was released from the assay solution (and the reaction stopped) by acidification. The assay mix consisted of 1.25 mM dithiothreitol, 1.9 mM putrescine, 50 mM sodium phosphate buffer, pH 6.8, and 9.6 μM [^{14}COOH]AdoMet (52 mCi/mmol) in a total volume of 250 μL. After addition of enzyme, the tubes were immediately capped with a rubber cap filter attached to a plastic well containing 0.3 mL of hyamine hydroxide. After incubation at 37 °C for 15 min, the reaction was stopped by injection of 0.3 mL of 5 M H_2SO_4 through the rubber cap. After an additional incubation of 30 min at 37 °C, the wells were removed and radioactivity was determined after the addition of 10 mL of Econofluor scintillation fluid. The assay was carried out under conditions where less than 5% of the substrate was used up and $^{14}CO_2$ production was linear with time for at least 30 min even when the lowest concentrations of substrate [^{14}COOH]-AdoMet were used.

Kinetic Studies. A three-component buffer (AMT buffer) containing 0.05 M sodium acetate, 0.05 M 2-(*N*-morpholino)-ethanesulfonic acid, and 0.1 M triethanolamine (*20*) was used for the AdoMetDC activity assay to ensure constant ionic strength throughout the pH range used. Each assay contained 40−4000 ng wild-type or C82A mutant protein. Each 250 μL reaction contained 1.25 mM dithiothreitol, 1.9 mM putrescine, and 9.6 μM [^{14}COOH]AdoMet (52 mCi/mmol) and varying concentrations of unlabeled AdoMet. Enzymatic activity was measured as the amount of $^{14}CO_2$ released per minute per milligram of protein. To determine the kinetic parameters k_{cat} and K_m, the initial velocities were measured at six AdoMet concentrations and the data were fitted to the Michaelis−Menten equation:

$$v = k_{cat}S/(S + K_m) \quad (1)$$

where v is the initial rate and S is the AdoMet concentration.

The k_{cat} and k_{cat}/K_m values were measured at various pH values. Plots of these values against pH were then constructed and fitted to (*21*).

$$x \text{ or } y = C/[(1 + H/K_a)(1 + K_b/H)] \quad (2)$$

$$x \text{ or } y = C/[(1 + H/K_a)] \quad (3)$$

where x is k_{cat}, y is k_{cat}/K_m, H is the proton concentration, K_a and K_b are the ionization constants of the groups involved in the enzymatic reaction, and C is the pH-independent value of either k_{cat} or k_{cat}/K_m. Equation 2 provided the best fit for curves of data from wild-type AdoMetDC, which showed two independent slopes, and eq 3 provided the best fit for curves of data from mutant C82A, which showed only one slope. KaleidaGraph (Abelbeck Software) was used to fit the curves by the Levenberg−Marquardt nonlinear fitting algorithm. The parameters were determined at each pH with six substrate concentrations and at least triplicate determinations at each substrate concentration. The standard deviation never exceeded 10% of the average value.

IAA Inhibition and Labeling of AdoMetDC. Wild-type (1.6 μg) or C82A mutant (160 μg) AdoMetDC proteins were incubated with 3.2 mM IAA in AMT buffers at various pH in a total volume of 160 μL. The reactions were carried out in the dark at 37 °C and were terminated by the addition of excess of DTT at various time periods. The remaining activity of 40 ng of wild-type or 4 μg of C82A IAA-treated protein were measured in the standard AdoMetDC assay described above. The proteins in the control experiments were treated similarly except that no IAA was added.

To measure the incorporation of IAA into the purified proteins, wild-type and C82A mutant protein were treated with 3.2 mM iodo[2-^{14}C]acetic acid (25 mCi/mmol) in AMT buffer at various pH values at 37 °C in the dark. Aliquots of labeled proteins were taken at various time periods and precipitated with 10% trichloroacetic acid (TCA). The pellet was washed 8 times with 10% TCA and then resuspended in BCS scintillation fluid (Amersham) to count the total radioactivity. The amount of the incorporated IAA was calculated by using a counting efficiency of 0.565 determined simultaneously with standard ^{14}C-benzoic acid. The protein content in the aliquots were verified by using the Bio-Rad dye-binding assay kit. The incorporation of IAA into AdoMetDC was visualized by resolving the ^{14}C-labeled proteins with SDS−PAGE and subsequent autoradiography. The time course of the labeling was examined by incubating wild-type and C82A mutant proteins with iodo[2-^{14}C]acetic acid at pH 9.2. At 0, 5 15, 30, and 60 min, aliquots of the reaction mix were taken out and precipitated by 10% TCA and their radioactivity was measured. The molar incorporation of IAA was calculated and plotted against the amount of protein used in each assay (Figure 5c). A best-fit line was calculated for each set of data by a least-squares method and the slopes were used to determine the molar ratio of incorporated IAA.

Substrate-Mediated Inactivation. The rate of substrate-mediated inactivation was measured by incubating 40 ng of wild-type or 4 μg of C82A proteins at 37 °C for various time periods with or without 0.4 mM AdoMet in the presence of 1.25 mM dithiothreitol, 1.9 mM putrescine, and 50 mM sodium phosphate buffer, pH 6.8. After the preincubation, AdoMetDC activity was measured in the standard assay. [^{14}COOH]AdoMet (53 mCi/mmol) was added to the mixtures that contained 0.4 mM AdoMet for the activity measurement. Both labeled and unlabeled AdoMet were added to the control assay mix prior to activity assay so that every reaction had the same amount of labeled and unlabeled substrate during the actual enzyme activity assay. The residual AdoMetDC activities were expressed as the percentage of the activity measured at zero time and the log values of these percentages was plotted against time. The line providing the best fit by the least-squares method was obtained by fitting the data with KaleidaGraph and the inactivation rate constant obtained from the slope of this line.

Peptide Sequencing. Both wild-type and C82A AdoMetDC were incubated at 37 °C for 90 min in the presence or absence of 0.4 mM AdoMet as described above. The proteins were then passed through a small G-25 column preequilibrated with 10 mM NaCl to remove components that interfere with the Edman degradation reaction. The proteins were then

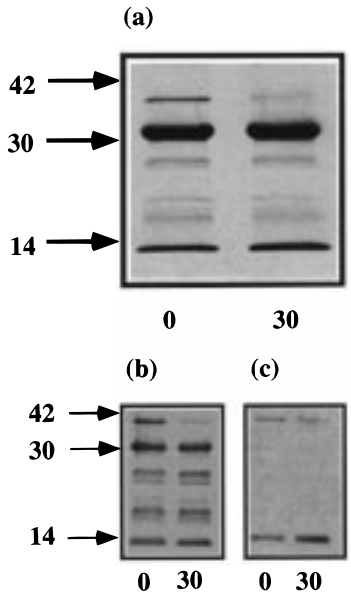

FIGURE 2: Purification of His-tagged human AdoMetDC. Panel a shows a Coomassie-stained SDS−polyacrylamide gel analysis of the purified wild-type protein. The molecular weight markers are shown on the left of the gel. The left lane is the freshly purified protein and the right lane is the same protein after incubation at 37 °C for 30 min. Each lane contains 30 μg of protein. Panels b and c show western blot analyses of the purified wild-type AdoMetDC. The preparations of AdoMetDC (unincubated and incubated for 30 min at 37 °C) were transferred onto PVDF membrane and blotted with rabbit anti-AdoMetDC antiserum (panel b) or mouse anti-His-tag antibody (panel c).

subjected to gas-phase peptide sequencing on an Applied Biosystems 477A protein sequencer (*5*, *11*).

Quantitation of AdoMetDC Protein. The amount of AdoMetDC having a pyruvoyl group was determined by measuring the binding of [^{35}S]dcAdoMet to the protein after reduction with sodium cyanoborohydride to reduce the Schiff base between the protein and the dcAdoMet, under the conditions previously described to give stoichiometric labeling of AdoMetDC (*18*).

RESULTS

Purification of His-Tagged Human AdoMetDC. A histidine tag was added to the N-terminus of wild-type and mutant human AdoMetDCs to facilitate purification via immobilized metal affinity chromatography. Three major protein bands were present in the purified AdoMetDC (Figure 2a). The apparent molecular mass of these three bands correlated well with the expected molecular mass values of the recombinant proenzyme, α subunit, and β subunit of human AdoMetDC (39.6, 30.6, and 8.9 kDa), respectively. Rabbit anti-human AdoMetDC antiserum recognized all three bands (Figure 2b). The 39 kDa band disappeared after the purified protein was incubated at 37 °C for 30 min. These results indicate that the 39 kDa band is the AdoMetDC proenzyme and that autocatalytic processing results in the disappearance of this band on both SDS−PAGE and Western blots. The anti-His-tag antibody only recognized the 39 and 9 kDa bands (Figure 2c), which is expected since the His-tag is present at the N-terminus of the proenzyme and the β subunit. Thus, it is clear that the 39, 31, and 9 kDa bands represent the proenzyme, α subunit, and β subunit, respectively.

In addition to the three AdoMetDC protein bands, five weak protein bands (approximately 23, 21, 16, 14, and 12 kDa) were observed (Figure 2a). The amount of protein in each of the bands was estimated by measuring the densitometry of the Coomassie blue-stained protein bands and the five extra peptides were found to consist of less than 5% of the total protein in the purified AdoMetDC. An MGBG-Sepharose affinity column, which has been used to purify AdoMetDC (*19*), was used to try to remove the five extra bands from the affinity-column-purified AdoMetDC with no success (data not shown). The rabbit anti-human AdoMetDC antiserum recognized all five extra bands seen on SDS−PAGE (Figure 2b). On the other hand, the anti-His-tag antibody did not recognize any of them (Figure 2c). Therefore, the five extra bands present in the purified AdoMetDC are very likely to be degradation products of AdoMetDC. Although they lack the N-terminal His-tag and should not bind to the metal affinity column, the five extra peptides might bind to one processed $\alpha\beta$ heterodimer of AdoMetDC and be copurified with intact enzyme.

The purified C82A protein showed an SDS−PAGE pattern similar to that of the wild-type protein (data not shown). The 39 kDa band that existed in the freshly purified C82A mutant AdoMetDC also disappeared after incubation of the protein at 37 °C for 30 min.

pH Effect on the Kinetic Parameters of Human AdoMetDC. The activity of the purified C82A mutant AdoMetDC was much lower than that of the wild-type enzyme but could easily be measured, although it was necessary to use 100-fold more of the mutant protein for each reaction in order to measure the enzyme activity accurately. The pH dependence of the k_{cat} and k_{cat}/K_m values for the C82A mutant was studied and compared to that of the wild-type enzyme in order to study the role of Cys-82 in the catalytic reaction (Figure 3). The values of the kinetic parameters were fitted to either eq 2 or eq 3 according to whether the shapes of pH profile curves showed one or two slopes. The results of this study are summarized in Table 1.

The pH dependency of k_{cat} (the first-order rate constant of the formation of the product) reflects the ionization state of the residues involved in the rate-determining step in the overall enzymatic reaction (*22*). The pH profile of k_{cat} of wild-type protein was a curve that showed an ascending limb with a slope of +1 and a descending limb with a slope of −1. This indicates that one group has to be unprotonated and another group has to be protonated for the activity of AdoMetDC. By fitting the data to eq 2, two p*K* values were obtained, 6.3 ± 0.2 and 7.8 ± 0.2. The group with p*K* of 6.3 must be unprotonated and the group with p*K* of 7.8 must be protonated to keep the enzyme active. It was very difficult to measure the activity of AdoMetDC at very low pH (pH < 5.6), so the possibility that another residue(s) needs to be unprotonated for activity cannot be ruled out.

The k_{cat} pH profile of C82A mutant was very different from that of the wild-type enzyme and the apparent k_{cat} value of this mutant was much lower than that observed for wild-type enzyme at all the pH values tested. The k_{cat} vs pH curve of C82A rose with a slope of +1. Above pH 7, the curve reached a plateau and stayed constant through pH 9.2. The presence of only one slope indicated only a single protonation event, and when the data were fitted to eq 3, a single p*K* of 6.0 ± 0.2 was obtained. This value is not significantly

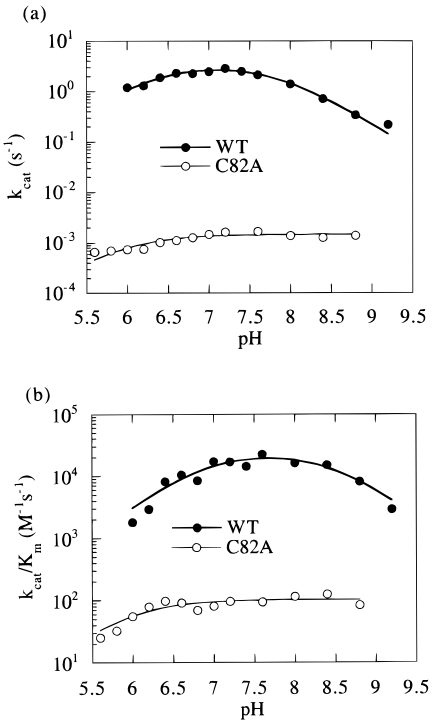

FIGURE 3: pH profiles of kinetic parameters k_{cat} and k_{cat}/K_m. The activity of wild type (WT; 40 ng/assay) and C82A (4 μg/assay) were measured at various pH values. The values of the kinetic parameters were determined as described in Materials and Methods and were plotted against the pH at which they were determined. The pH profile curves were fitted to either eq 2 or eq 3 according to their shape. See text for details. Panel a shows the pH profile of k_{cat} of wild type and C82A mutant, and panel b shows the pH profile of k_{cat}/K_m of wild type and C82A mutant.

different from the lower p*K* value of 6.3 obtained with the wild-type AdoMetDC ($p > 0.7$ by Student's *t* test). Thus, the pK_1 of the wild type is likely to represent the same ionization as that represented by the single p*K* value obtained for C82A. However, more importantly, C82A lacked the residue with a p*K* of 7.8 observed in wild type. This result suggests that Cys-82 may be the important residue involved in catalysis with p*K* around 7.8. The pH-independent value (*C*) of the apparent k_{cat} of C82A mutant was about 2000-fold lower than that of wild-type protein.

The k_{cat}/K_m value is the apparent second-order rate constant for the reaction of free substrate and free enzyme (*22*). The wild-type enzyme showed a bell-shaped curve ascending with a slope of +1 and descending with a slope of −1 (Figure 3b). Fitting the data to eq 2 revealed two p*K* values, 6.9 ± 0.2 and 8.5 ± 0.2. Since this process involves uncomplexed substrate and enzyme, the observed p*K* values should represent the p*K* values of important ionizations involved. However, none of the four p*K* values of AdoMet (*23*) are compatible with the two p*K* values observed in the wild-type protein k_{cat}/K_m pH profile. The two p*K* values observed on the k_{cat}/K_m curve of wild-type protein should therefore represent the ionization of the amino acid residues of the enzyme.

The k_{cat}/K_m vs pH curve of the C82A mutant displayed a different pattern from that of wild type. Although the k_{cat}/K_m values were much lower than those of wild-type protein, the ascending limb at the low-pH side still rose with a slope of +1. The curve reached a plateau at about pH 6.8 and stayed constant through pH 9.2. Upon fitting of the data to eq 3, the p*K* value on the low pH side for C82A was 6.0 ± 0.2. This value is significantly different from the pK_1 of 6.9 found for wild type ($p < 0.05$ by Student's *t* test). The lack of the second p*K* value for the mutant C82A suggests that Cys-82 may be the residue with a p*K* value of 8.5 that was observed on the descending limb of the k_{cat}/K_m curve for wild-type protein.

Inhibition of the Activity of AdoMetDC by IAA. IAA had a strong inhibitory effect on the activity of wild-type AdoMetDC (Figure 4a). The activity of the control, non-IAA-treated, wild-type enzyme remained relatively constant through the pH range 7−9.2 with a small decrease at pH 6. After being treated with IAA at 37 °C for 30 min, the activity of wild-type protein was significantly lower than control at all the pH values tested, but the greatest effect was seen at pH 9.2 (Figure 4a). At pH 6.0 and 7.0, IAA-treated wild-type protein had about 60−65% of the activity of the control untreated protein activity. At pH 9.2 only about 5% of the AdoMetDC activity remained after treatment. In contrast, IAA did not have any effect on the activity of C82A mutant and the activities of treated and untreated C82A mutant protein were almost the same at all the pH values tested.

The inactivation by IAA was also time-dependent. At pH 9.2, a significant decrease in activity of wild-type protein was observed between 2 and 30 min treatment (Figure 4b). After 60 min treatment, essentially no activity of wild-type protein remained. The activity of non-IAA-treated control experiments showed no changes during the 60 min incubation at 37 °C.

Since the inactivation of AdoMetDC was more efficient at high pH, pH 9.2 was chosen to study the covalent interaction of iodo[2-^{14}C]acetic acid with the protein. Figure 5a shows the image of the autoradiogram of wild-type and C82A protein after incubating with iodo[2-^{14}C]acetic acid for 30 min. Both wild type and C82A mutant were labeled and the radioactivity was almost all in the 31 kDa α subunit. The incorporation of IAA was time-dependent and a significant difference of IAA incorporation between wild type and C82A was evident within the first 5 min of incubation (Figure 5b). The incorporation of IAA into wild-type AdoMetDC continued to go up from 15 to 60 min while the incorporation for C82A reached a plateau at about 30 min (Figure 5b).

Values of the total incorporation of IAA into the wild-type and C82A mutant AdoMetDC were obtained by carrying out the reaction at a range of protein concentrations and plotting the best-fit line of incorporation against protein using the least-squares method (Figure 5c). The maximal incorporation was 2.7 mol of IAA/mol of $\alpha\beta$ dimer (95% confidence interval of 2.3−3.0) for wild-type AdoMetDC protein and 1.8 mol of IAA/mol of $\alpha\beta$ dimer (95% confidence interval of 1.5−2.0) for the C82A mutant.

Substrate-Mediated Inactivation. Incubation at 37 °C for 60 min in the absence of substrate had very little effect on the activity of either wild-type or C82A mutant human AdoMetDC (Figure 6). Preincubation with AdoMet substrate caused a significant decrease in enzyme activity of both wild-type and C82A mutant AdoMetDC enzymes (Figure 6). However, C82A showed a faster rate of inactivation than that of wild type. Linear plots were obtained of the log of the activity remaining against time for both wild-type and

Table 1: Summary of the Kinetic Parameters Obtained from pH Profile Studies

protein used	parameter[a]	eq	*C* value[b]	pK_1	pK_2
wild-type AdoMetDC	apparent k_{cat} (s^{-1})	2	3.6 ± 0.25	6.3 ± 0.2	7.8 ± 0.2
C82A AdoMetDC	apparent k_{cat} (s^{-1})	3	$(1.47 \pm 0.07) \times 10^{-3}$	5.9 ± 0.2	*c*
wild-type AdoMetDC	apparent k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	2	$(2.57 \pm 0.40) \times 10^{4}$	6.9 ± 0.2	8.5 ± 0.2
C82A AdoMetDC	apparent k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	3	$(1.03 \pm 0.069) \times 10^{2}$	6.0 ± 0.2	*c*

[a] The values of the parameters were obtained by fitting data to equations indicated. The R^2 (coefficient of determination, which is the fraction of the total variance of k_{cat} or k_{cat}/K_m that is explained by the model and is a measure of the goodness of fit of the nonlinear regression curve) values for all curve-fitting were greater than 0.9. [b] *C* is the pH-independent value of either k_{cat} or k_{cat}/K_m as described under Materials and Methods. Values are shown in the format of mean ± SD. [c] Indicates that the value does not exist for this protein.

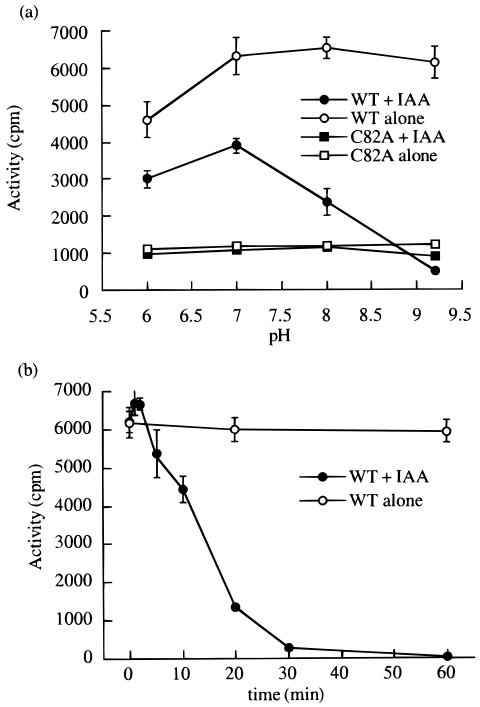

FIGURE 4: Inactivation of AdoMetDC by iodoacetic acid. Panel a shows the pH dependency of the inactivation by IAA of wild-type (WT) and C82A mutant AdoMetDCs after treatment with 3.2 mM IAA for 60 min. The proteins in the control experiments were treated the same way except that no IAA was added to the incubation. Each point represents the mean of at least three independent assays with the bar ± SD of the results. Panel b shows the time course of the inactivation with IAA in AMT buffer, pH 9.2.

C82A mutant AdoMetDCs (Figure 6). This suggests that the inactivation is pseudo-first-order for both enzymes and the slopes of the linear plots therefore represent the inactivation rate. The mean of the inactivation rates of wild type and C82A mutant were 0.0098 min^{-1} (95% confidence interval of 0.0073−0.0117) and 0.0175 min^{-1} (95% confidence interval of 0.0154−0.0195), respectively.

To test if the substrate-mediated inactivation of AdoMetDC was due to transamination, the protein was taken after incubation with the substrate for 90 min and the amino-terminal sequence was determined for 5 cycles using a gas-phase sequencer. [Previous studies have shown that the presence of pyruvate at the amino terminus of the α subunit prevents sequencing by Edman degradation and that the transamination of the pyruvate, forming alanine, allows the sequencing to proceed and provides a measure of the amount of the transaminated form of the enzyme present (*5*, *7*, *11*, *24*)]. Since the α and *β* subunits were not separated prior to this analysis, two amino acids were released in every cycle.

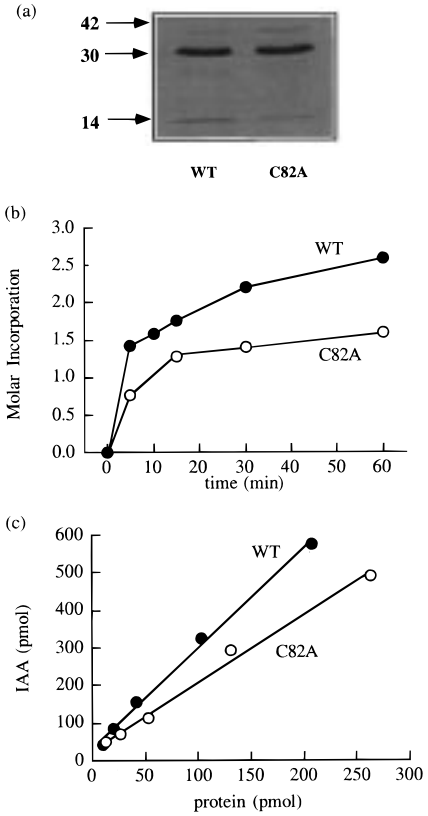

FIGURE 5: Incorporation of iodo[2-^{14}C]acetic acid into AdoMetDC. All the reactions were carried out with 3.2 mM iodo[2-^{14}C]acetic acid (25 mCi/mmol) in AMT buffer, pH 9.2 at 37 °C. Panel a shows SDS−PAGE analysis of the labeled protein obtained after wild-type (WT) or C82A mutant proteins (10 *μ*g each) were treated with iodo[2-^{14}C]acetic acid at 37 °C for 30 min in a total volume of 50 *μ*L. The proteins were resolved by SDS−PAGE on a 12.5% gel. The gel was dried and exposed to X-ray film. The molecular weight markers are shown on the left side of the autoradiogram. Panel b shows the time course of the incorporation of iodo[2-^{14}C]acetic acid into wild-type (WT) and C82A mutant AdoMetDC. Aliquots (5 *μ*g of each protein) were treated with iodo[2-^{14}C]acetic acid at 37 °C in a total volume of 50 *μ*L. Each point represents the average of at least three independent measurements. Panel c shows the curves used for the determination of molar incorporation of IAA into AdoMetDC. Varying amounts of wild-type (WT) and C82A protein were treated with 3.2 mM iodo[2-^{14}C]acetic acid (25 mCi/mmol) in AMT buffer, pH 9.2, at 37 °C for 1 h in a total volume of 50 *μ*L. The IAA incorporation values (picomoles) were plotted against the amount of proteins (picomoles) in the reactions. Each point represented the average of triplicate reactions. The data were fitted to linear equations (for wild type, $R = 0.998$, and for C82A, $R = 0.996$, where *R* is the correlation coefficient).

These amino acids corresponded to the sequences expected from the *β* and the transaminated form of the α subunits (MRGSH- and ASMFV-, respectively). With the wild-type AdoMetDC, the relative amount of the latter sequence was

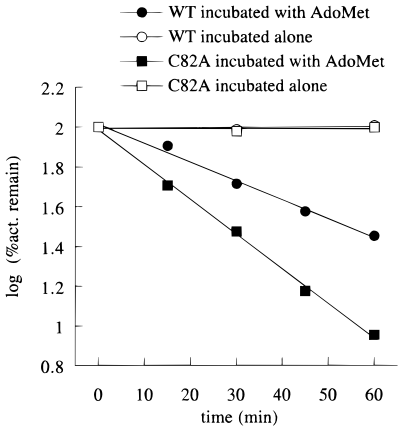

FIGURE 6: Substrate-mediated inactivation of AdoMetDC. Both wild-type (WT) and C82A mutant AdoMetDC were incubated with or without 0.4 mM AdoMet as described in Materials and Methods. Aliquots were taken at time points indicated on the graph and assayed for remaining AdoMetDC activity. Activities at each time point were expressed as percentage of the activities at time zero. The log values of the remaining activity were plotted against time and fitted to a linear equation. The values on the graph are means of at least three independent assays. Standard deviations never exceed 10% of the means.

very low in the enzyme incubated without substrate and was increased about 20-fold after incubation with AdoMet. Averaging the ratio of the relevant amino acids at each cycle, the results indicated that the amount of wild-type AdoMetDC was 5.2% ± 3.9% in the transaminated form prior to reaction and virtually 100% (110% ± 15%) was in the transaminated form after reaction. This provides conclusive evidence that the inactivation of human AdoMetDC does occur by conversion of the pyruvate prosthetic group to alanine. Although differences between the efficiencies of cleavage for different amino acids in the sequencing procedure may affect the calculation, this does not affect the finding that there is an approximately 20-fold increase in response to incubation with substrate since the same cleavages occur for both samples. The small amount of recombinant wild-type AdoMetDC in the transaminated form is, however, in agreement with previous reports that this form can be detected in recombinant AdoMetDC from several species (*7*, *11*, *24*). The transaminated enzyme may be produced during growth of the bacteria expressing human AdoMetDC.

When the C82A mutant AdoMetDC was examined in the same way, a much higher percentage of the protein (87% ± 34%) was found to be in the transaminated form prior to reaction with substrate. There was a increase in this value to 95% ± 27% after reaction with substrate, which is consistent with transamination. The much higher percentage of the untreated C82A protein in the transaminated form also provides strong support for the hypothesis that this mutant is much more readily transaminated. However, the sequencing method is not sufficiently accurate to provide statistically significant proof that loss of the pyruvate group accounts for the substrate-mediated inactivation.

A more accurate method to determine the percentage of the C82A mutant protein containing pyruvate at the active site was therefore used. In this method, the pyruvate at the active site is titrated by the reaction with dcAdoMet and sodium cyanoborohydride (*18*). Previous studies have shown that this procedure provides stoichiometric labeling of AdoMetDC at the active site, which is due to the covalent attachment of the dcAdoMet produced by the cyanoborohydride-mediated reduction of the Schiff base linkage at the pyruvate group (*18*). When [^{35}S]dcAdoMet and sodium cyanoborohydride were allowed to react with recombinant wild-type and C82A mutant AdoMetDCs in our experiments, wild-type AdoMetDC incorporated (113 ± 23) × 10^6 dpm/μg of protein under these conditions whereas the C82A mutant incorporated (33.2 ± 1.9) × 10^3 dpm/μg of protein. This indicates that only 3% of the recombinant C82A AdoMetDC was actually in the potentially active pyruvoyl form. It is very likely that the rest of it was transaminated. The transamination of the recombinant C82A AdoMetDC presumably occurs during the growth of the bacteria and is totally consistent with the rapid inactivation of the protein described above.

DISCUSSION

Our results show clearly that Cys-82 is a very important residue in the catalytic mechanism of AdoMetDC. The structure of this enzyme has very recently been solved by X-ray crystallography (*25*) and is consistent with this hypothesis since Cys-82 is located in the active-site cleft and is close to the pyruvate prosthetic group. As discussed earlier, Cys-140 in *E. coli* AdoMetDC, the counterpart of human Cys-82, is very likely to be at or near the active site of this enzyme (*15*, *16*). The C82A mutation in human AdoMetDC dramatically decreased the activity of AdoMetDC and led to a large increase in the amount of the enzyme that was transaminated to form alanine from the pyruvate at the active site. Since our studies show that only 3% of the C82A AdoMetDC is in the active pyruvoyl form, the apparent k_{cat} value for this protein found in the kinetic study must be increased accordingly. These apparent turnover numbers for wild type and C82A mutant are 2.43 s^{-1} and 0.00127 s^{-1}, respectively (see above). Correcting the latter value for the amount of active enzyme gives a value of 0.0442 s^{-1}. The ratio of the turnover to inactivation for wild-type AdoMetDC when incubated with AdoMet is therefore about 14 900, while for C82A mutant this ratio is only 143. Thus, the inactivation occurs much more frequently for the C82A mutant than for wild-type AdoMetDC under the reaction conditions tested. For *E. coli* AdoMetDC, the ratio of turnover to inactivation was estimated to be about 6600 (*15*), which is comparable with that of wild-type human AdoMetDC.

The relatively common occurrence of incorrect protonation of the pyruvoyl group leading to transamination of the AdoMetDC is likely to account for the high percentage of the transaminated form of the protein found in the recombinant C82A AdoMetDC preparations. The smaller but detectable amount of the transaminated wild-type protein is also likely to be produced during growth of the bacteria expressing the recombinant protein. On the basis of the rates of inactivation shown in Figure 6, it may be questioned why the fraction of AdoMetDC obtained in the transaminated form is not even higher. However several factors, which were not investigated, could contribute to this, including (i) the brief period (3 h) of IPTG-induced synthesis of the recombinant protein, (ii) the possibility of rapid degradation of the transaminated form, (iii) the less efficient purification of the transaminated form, and (iv) limitation of the availability of

AdoMet or dcAdoMet due to a limited capacity of AdoMet synthetase, compartmentalization, or binding to other proteins.

The pH profiles of the kinetic parameters k_{cat} and k_{cat}/K_m of the wild-type and the C82A mutant AdoMetDC are also consistent with a key role for Cys-82. Both the k_{cat} and k_{cat}/K_m pH profiles of C82A mutant lack the descending limbs observed in wild-type profiles (Figure 3). The simplest and most probable explanation for this difference is that Cys-82 is involved in both substrate binding and catalysis and must be protonated to keep the enzyme in an active state. The apparent p*K* value (from k_{cat}/K_m pH profile) of Cys-82 would then be 8.5 ± 0.2. Another possibility is that the alteration of Cys-82 to Ala produces a conformational change in the protein that raises the p*K* of another residue so that it is completely outside the range tested.

A significant shift of pK_1 was also observed in the C82A mutant in the k_{cat}/K_m vs pH profile (Table 1). On the acidic side of the profiles, wild-type protein shows an important ionization with p*K* around 6.9 ± 0.2, while C82A mutant shows a p*K* around 6.0 ± 0.2. One explanation could be that mutation C82A causes a conformational change that results in the decrease of the p*K* value of the unidentified residue from 6.9 to 6.0. Another explanation could be that the change caused by the C82A mutation is so dramatic that the slow step of the catalysis of decarboxylation of AdoMet changes. If this is the case, the p*K* values observed in the k_{cat}/K_m pH profiles of wild type and C82A mutant actually represent two different residues. The role for the residue with the acidic p*K* value is unknown but it may act to remove the proton from the amino group of the AdoMet substrate to allow formation of the Schiff base.

The importance of Cys-82 in the action of AdoMetDC is also demonstrated by the studies with IAA, which reacts specifically with cysteine residues. IAA inhibits the activity of wild-type AdoMetDC but not the C82A mutant (Figure 4), indicating that Cys-82 is the residue that is responsible for the IAA inactivation of the wild-type AdoMetDC. Although there are seven Cys residues in wild-type AdoMetDC, the results show that the molar ratio of incorporated IAA is 2.7 for wild-type protein and 1.8 for C82A mutant (Figure 5), suggesting that most are unavailable to react rapidly with IAA. The difference of molar incorporation of IAA into AdoMetDC between wild type and C82A mutant of 0.9 suggests that Cys-82 reacts readily and virtually completely with the reagent. The inhibition of wild-type AdoMetDC by IAA was very efficient at pH values higher than 7, suggesting that there is a Cys residue(s) with a basic p*K* that is very important for AdoMetDC activity and that modification of this Cys residue by IAA results in inhibition of the enzyme activity.

These results are all consistent with the hypothesis that Cys-82 may be the essential proton donor for the AdoMetDC reaction. If this is the case, then another residue must take over this function in the C82A mutant, albeit much more slowly as reflected in the apparent k_{cat} values. The incorrect positioning of this residue may lead to the high frequency of incorrect protonation of the pyruvate moiety rather than the α-carbon of the product leading to transamination (see Figure 1, transamination pathway). Studies of mutants of histidine decarboxylase, another pyruvoyl enzyme, have shown that alteration of Glu-197 to Asp led to a protein in which activity was lost after a few turnover cycles, suggesting that Glu-197 may act as the proton donor in that case (*12*). Another explanation for our results, which cannot be ruled out on the basis of experimental data, is that Cys-82 is involved in the formation of the immediate proton donor but is not itself actually the residue that carries out this function. Residue Glu-11, which is located very close to Cys-82 (*25*) and is essential for AdoMetDC activity (*26*), is a possible candidate for such interaction.

Since AdoMetDC is a very important enzyme in polyamine biosynthesis, several AdoMetDC inhibitors have been developed as chemotherapeutic drugs in treating various cancers and parasite infections (*3*, *27*). Many of these inhibitors are not effective in vivo or have serious side effects. Hence, developing more specific and more potent AdoMetDC inhibitors is highly desirable. A detailed knowledge of the mechanisms of the processing and catalysis of AdoMetDC is absolutely required for the development of new inhibitors. Most AdoMetDC inhibitors are targeted to the pyruvate prosthetic group. The results of the current study support an alternative target for the inhibitor design, where interactions of an inhibitor with Cys-82 of human AdoMetDC may promote transamination of the pyruvate and irreversible inactivation of AdoMetDC. In fact, studies on one of the AdoMetDC inhibitors, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (AbeAdo) have shown that stimulating the transamination is a viable approach for AdoMetDC inhibition. AbeAdo is a highly potent drug in treating *Trypanosoma brucei* infection (*28*) and malaria (*29*). Although designed as an enzyme-activated irreversible inhibitor (*30*, *31*), AbeAdo actually causes very high frequency of transamination, rather than the originally anticipated covalent modification of AdoMetDC (*7*).

Although AdoMetDC inhibitors are generally non-species-specific, variability of susceptibility to different inhibitors does exist among various species (*3*). For example, the *T. brucei* AdoMetDC has been shown to have a more sterically hindered active site than that of mammalian enzymes and is more susceptible to inhibition by AdoMet analogues with smaller side chains (*32*). Thus, detailed structure−function studies of specific AdoMetDC enzymes may also turn out to be critical in inhibitor design.

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