Chemical Modification and Site-directed Mutagenesis of Cysteine Residues in Human Placental S-Adenosylhomocysteine Hydrolase*

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Human placental S-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) was inactivated by 5,5-dithiobis(2-nitrobenzoic acid) following pseudo-first-order kinetics. Modification of three of the 10 cysteine residues per enzyme subunit resulted in complete inactivation of the enzyme. The three modified cysteine residues were identified as Cys113, Cys195, and Cys421, respectively, by protein sequencing after modification with [1-35S]iodoacetamide. Of the three modifiable cysteines, Cys113 and Cys421 could be protected from modification in the presence of the substrate adenosine (Ado), which also protected the enzyme from inactivation. On the other hand, Cys421 was not protected by Ado, and modification of Cys421 alone did not affect the enzyme activity. To verify whether some of these cysteine residues are important for the enzyme catalysis, these three cysteine residues were replaced by either serine or aspartic acid using site-directed mutagenesis. Mutants of both Cys113 (C113S and C113D) and Cys421 (C421S and C421D) had enzyme activities similar to that of the wild-type enzyme, and only slight changes were observed in the steady-state kinetics measured in both the synthetic and hydrolytic directions. However, mutants of Cys195 (C195D and C195S) displayed drastically reduced enzyme activities, and kcat values were only 7 and 12% of that of the wild-type enzyme, respectively, resulting in a calculated loss in binding energy (∆G) of approximately 1 Kcal/mol. The Cys195 mutations were capable of catalyzing both the 3'-oxidative and 5'-hydrolytic reactions, as evidenced by the reduction of E-NAD+ to NADH and formation of the 5'-hydrolytic product when incubated with (E)-5,6-didehydro-6'-deoxy-6'-chlorohomoadenosine at rates comparable with those catalyzed by the wild-type enzyme. However, mutations of the Cys195 severely altered the 3'-reduction potential as evidenced by the drastic reduction in the rate of [2,8-3H]Ado release from the E-NADH[(E)-5,6]-keto-Ado complex. Circular dichroism studies of the Cys195 mutants confirmed that the observed effects are not due to changes in secondary structure. These results suggested that the Cys195 is involved in the catalytic center and may play an important role in maintaining the 3'-reduction potential for effective release of the reaction products and regeneration of the active form (NAD+ form) of the enzyme; the Cys113 is located in or near the substrate binding site, but plays no role beneficial to the catalysis; and the Cys421 is a nonessential residue, which also explains why Cys421 does not occur in any other known AdoHcy hydrolases.

S-Adenosyl-L-homocysteine (AdoHcy) catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and l-homocysteine (Hcy) (1). Because of its important role in regulating biological methylation reactions (2), AdoHcy hydrolase has been an attractive target for the design of antiviral (3–6), antiparasitic (7), and antiarthritic agents (8). AdoHcy hydrolase has been cloned from a number of different sources, including Homo sapiens (9), Rattus species (10), Plasmodium falciparum (11), Rhodobacter capsulatus (12), Triticum aestivum,2 Catharanthus roseus,3 Petroselinum crispum (15), Leishmania donovani (16), Dictyostelium discoideum (17), and Caenorhabditis elegans (18). The amino acid sequences of the cloned AdoHcy hydrolases have been deduced from their cDNA sequences. Comparison of the amino acid sequences from these species shows a remarkable degree of conservation ranging from 64% identity between human and Rhodobacter capsulatus (12) to 97% identity between human and rat (9). All of the cloned AdoHcy hydrolases are tetramers with Mw values between 180,000 and 200,000. AdoHcy hydrolase contains four tightly bound molecules of NAD+ and consists of structurally identical subunits that are catalytically equivalent and functionally independent (19).

The mechanism by which AdoHcy hydrolase catalyzes the conversion of AdoHcy to Ado and Hcy, as well as the reverse reaction, has been elucidated by Palmer and Abeles (20). In the hydrolytic direction, the first step involves oxidation of the 3'-hydroxyl group of AdoHcy (3'-oxidative activity) by enzyme-bound NAD+ (E-NAD+), followed by β-elimination of l-Hcy to give 3'-keto-4',5'-didehydro-5'-deoxy-Ado. Michael addition of water to the 5'-position of this tightly bound intermediate (5'-hydrolytic activity) affords 3'-keto-Ado, which is then reduced by enzyme-bound NADH (E-NADH) to Ado (3'-reduction activity). In this case, the 5'-hydrolytic activity depends upon the 3'-oxidative activity.

More recently, specific inhibitors that utilize the 3'-oxidative activity and substrates that utilize the 5'-hydrolytic activity

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1 The abbreviations used are: AdoHcy, S-adenosyl-L-homocysteine; Ado, adenosine; Hcy, homocysteine; AdoMet, S-adenosyl-L-methionine; E-NAD+, enzyme-bound NAD+; E-NADH, enzyme-bound NADH; E-NAD+, NAD+ form of AdoHcy hydrolase; E-NADH, NADH form of AdoHcy hydrolase; EDDFHA, (E)-5,6-didehydro-6'-deoxy-6'-fluorohomoadenosine; EDDHIHA, (E)-5,6-didehydro-6'-deoxy-6'-chlorohomoadenosine; IPTG, isopropyl-β-D-galactopyranosid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DCHHA, 6'-deoxy-6'-chloro-5'-hydroxymethionine; Ade, adenosine; HPLC, high performance liquid chromatography; TPCK, L-1-tosyl-amido-2-phenylethyl chloromethyl ketone.

2 K. D. Richards and R. C. Gardner, GenBank™/EMBL accession number L11872.

3 G. Schroeder, A. Waitz, M. Hotze, and J. Schroeder, GenBank™/EMBL accession number Z26881.
have been developed for AdoHcy hydrolase (21–23). Some of these substances, such as (E)-5,6-didehydro-6-deoxy-6′-fluorohomoAdo (EDDFHA) and (E)-5,6-didehydro-6-deoxy-6′-chlorohomoAdo (EDDCHA), have been found to be valuable tools for use in dissecting the cause of AdoHcy hydrolase inactivation associated with site-directed mutagenesis or chemical modification (24) since, in this case, the 5'-hydrolytic activity is not dependent on the 3'-oxidative activity (23).

In contrast to the extensive studies on mechanisms of the enzyme catalysis and the enzyme inactivation, little is known about the structural features of the active site of AdoHcy hydrolase. To date, attempts at crystallization of this enzyme for x-ray studies have been unsuccessful. Therefore, efforts to identify residues in the active site of AdoHcy hydrolase have been made by several laboratories using alternative approaches, including chemical modification (25–28), affinity labeling (29, 30), limited proteolysis digestion (31), and site-directed mutagenesis (24, 32). Accumulated information from these studies has provided insights into the structural features of the enzyme needed for catalysis and inactivation and may ultimately lead to the rational design of more potent inhibitors of AdoHcy hydrolase.

In this study, we have used a combination of chemical modification and site-directed mutagenesis to identify a cysteine residue that is critical to the catalytic function of human placental AdoHcy hydrolase and to elucidate its possible role(s) in the mechanism of the enzyme action.

**EXPERIMENTAL PROCEDURES**

**Materials**—5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), iodoacetamide, trypsin (EC 3.4.21.4) (TPCK-treated, type XIII), and calf intestinal Ado deaminase (EC 3.5.4.4) were from Sigma. [1-14C]Iodoacetamide (11.6 mCi/mmol) and [2,8-3H]Ado (50 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). The oligonucleotide-directed in vitro mutagenesis system version 2.1 (Sculptor™), and Escherichia coli strain TG1 were from Amersham Corp. T4 DNA ligase, alkaline phosphatase, and Wizard™ miniprep DNA purification system were purchased from Promega (Madison, WI). DNA sequenase version 2.0 was from U. S. Biochemical Corp. Oligonucleotides were synthesized by Cruachem (Sterling, VA). [α-32P]SdATP was from DuPont NEN. Isopropryl-β-D-galactosidase (IPTG) was purchased from Fisher. E. coli JM109 strain containing plasmid pPR0Kcd20 encoding the recombinant human placental AdoHcy hydrolase was provided to our laboratory by Dr. Michael S. Hershfield (Duke University, Durham, NC). EDDCHA was provided by Dr. Morris Robins (Brigham Young University, Provo, UT).

**Overexpression and Purification of Wild-type AdoHcy Hydrolase**—Overexpression and purification of wild-type AdoHcy hydrolase was carried out in the procedures described previously (24, 33). Briefly, E. coli JM109 carrying the expression vector pPR0Kcd20 for recombinant human placental AdoHcy hydrolase were grown in 2 × YT medium and induced with 1 mM IPTG. The cell-free extract was used for the enzyme purification through the following steps: DEAE-cellulose ion exchange (batch method), ammonium sulfate precipitation (80%), gel filtration on Sephacryl S-300 column, and ion exchange on DEAE-Sephacore column. The protein concentration was determined by the method of Bradford (34) using bovine serum albumin as a standard, and the subunit $M_s$ was used to calculate the molarity of enzyme solutions.

**Assays for Enzyme Activities**—The assay of AdoHcy hydrolase activity in the synthetic direction was performed as described previously (21). This assay measures the rate of formation of AdoHcy from Ado and Hcy using HPLC. One unit of enzyme activity is defined as the amount of enzyme that can synthesize 1 μmol of Hcy formed (35). The blank was taken against a sample containing all other components except the enzyme. One unit of the enzyme activity is defined as the amount of enzyme that can hydrolyze 1 μmol of AdoHcy/min.

The assay of the 5'-hydrolytic activity of AdoHcy hydrolase toward EDDCHA was conducted by mixing 150 μl of enzyme solution (14.2 μm) in buffer containing 50 μl of 800 μCi EDDCHA. The reaction mixture was incubated at 37°C for various times, and the reaction was terminated by addition of 15 μl of 5 N HClO$_4$. After centrifugation, the supernatant was analyzed for the 5'-hydrolytic product 6′-deoxy-6′-chlorohomoAdo (DCHHA) by HPLC using a C18 reverse-phase column as described previously (23). The data were fitted to Equation 1,

$$P = (k_h/k_e)E_0(1 - e^{-kt})$$

where $P$ is the product DCHHA; $k_h$ is the rate constant of the 5'-hydrolytic reaction; $k_e$ is the rate constant of the enzyme inactivation, which is equal to the rate constant of the 3'-oxidative reaction; $E_0$ is the concentration of initial enzyme; and $k_{app}$ is the apparent rate constant of enzyme inactivation at this substrate concentration. Details of this equation have been reported previously by our laboratory (23).

The assay of the 3'-oxidative activity toward EDDCHA was performed under the same conditions as that for the 5'-hydrolytic activity assay except that the reaction was stopped by addition of 3 volumes of 95% ethanol to release NAD$^+$/NADH from the enzyme. After centrifugation, the supernatant was analyzed for the remaining NAD$^+$ contents by HPLC (30). Data were fitted to an exponential decay equation using the Ultrafit fitting program (Ultratfit, Cambridge, United Kingdom).

**Enzyme Inactivation by DTNB—DTNB stock solution (10 mM) was prepared in 0.1 M sodium phosphate buffer, pH 7.0. AdoHcy hydrolase (1.5 μg) was incubated with various concentrations of DTNB in a cuvette containing 3 ml of 0.1 M sodium phosphate buffer, pH 8.0, and 1 mM EDTA at 25°C. At intervals, an aliquot (30 μl) was removed from the reaction mixture and added to 470 μl of the enzyme assay mixture to measure the remaining enzyme activity in the synthetic direction. Controls containing no modification reagent were run concurrently at any given time, and the residual activity was calculated relative to the appropriate control.

The number of DTNB-modified cysteine residues was determined by measuring the absorbance at 412 nm on an HP 8452 diode array spectrophotometer using an extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$ for TBN.

**Incorporation of [1-14C]Iodoacetamide into AdoHcy Hydrolase**—For specific modification of cysteine residues located in the substrate binding site of the enzyme (15), 200 μl of 3 ml of 14C-labeled iodoacetamide (15.5 mCi/mmol) was added to the enzyme solution of the DTNB reaction mixture. The radioactivity incorporated into the enzyme was determined by gel filtration on a Sephadex G-50 spin column equilibrated with 0.1 M Tris-HCl buffer, pH 8.9, and was terminated by passing the reaction mixture through the spin column. The radioactivity incorporated into the enzyme was determined by liquid scintillation counting using a complete counting mixture 3a70B (Research Products International Corp., Mount Prospect, IL) after the modified protein was denatured with 8 M urea and passed through the spin column, which had been equilibrated with 1 M urea in the buffer A.

For specific modification of cysteine residues located outside of the substrate binding site, AdoHcy hydrolase (1.5 mg) was dissolved in 0.5 μl of buffer A containing 700 μM Ado. After incubation at 37°C for 10 min, the mixture was transferred to 0.5 ml of 0.2 M Tris-HCl buffer, pH 8.9. To the Ado-protected enzyme was then added 200 μl of 10.2 mM [1-14C]Iodoacetamide, and the mixture was incubated at 25°C in the dark for 1 h and was terminated by passing the reaction mixture through the spin column. The radioactivity incorporated into the enzyme was determined by liquid scintillation counting using a complete counting mixture 3a70B (Research Products International Corp., Mount Prospect, IL) after the modified protein was denatured with 8 M urea and passed through the spin column, which had been equilibrated with 1 M urea in the buffer A.

**Isolation and Identification of [1-14C]Carboxymethylated Peptides**—The specific radiolabeled AdoHcy hydrolases obtained from the procedures described above were heated at 100°C for 3 min in the presence of 8 M urea. To the denatured protein, dithiothreitol was added to a final concentration of 1 mM and incubated at 50°C for 10 min. After cooling
to room temperature, the protein was incubated with unlabelled iodacetate (10.2 mM) at 25°C for 15 min. The urea concentration was then reduced to 1 M by passing through the spin column equilibrated with 0.4 M NH₄HCO₃, pH 8.4, containing 1 M urea. A freshly prepared solution of TFCP-trypsin was added to give an enzyme-to-substrate ratio of 1:50 (by weight). After incubation for 5 h at 37°C, a second addition of the same amount of trypsin was made and the digestion was continued for another 5 h. After lyophilization, the trypsin digest was dissolved in 0.1% trifluoroacetic acid, and the peptides were analyzed on a Vydac C18 protein and peptide column (Vydac 218TP54, C18, 5 μm, 4.6×25 cm). The solvent system consisted of solvent I (0.1% trifluoroacetic acid) and solvent II (80% CH₃CN, 20% H₂O, 0.07% trifluoroacetic acid). The initial conditions were 2% solvent II with a linear gradient to 70% solvent II over 120 min at a flow rate of 0.5 ml/min. The UV absorbance of the eluted peptides was monitored at 220 nm. The radioactivity in the fractions collected (0.5 ml) was measured by liquid scintillation counting. Peptide peaks containing major radioactivity were collected and concentrated by Speed-Vac and rechromatographed on the same column using the initial conditions of 20% solvent II with a linear gradient to 60% solvent II over 60 min or 10% solvent II with a linear gradient to 40% solvent II over 60 min. Detection of peptides and measurement of radioactivity were the same as described above except that fractions were collected manually peak by peak.

The isolated peptides were sequenced by automated Edman degradation on an Applied Biosystems 473A protein sequencer in the Biotechnology Laboratory at Kansas State University, Manhattan, KS. At each sequencing cycle, the washings from the conversion flask and eluate from the HPLC column were collected and combined for determination of radioactivity.

Site-directed Mutagenesis—The EcoRI fragment from the pPROKci20 plasmid was subcloned into M13mp19, and the mutants were generated in this vector using Amersham’s Spoolit™ in vitro mutagenesis kit, which is based on the method of Taylor et al. (36), exploiting the inability of Ncol to cleave a thio-containing DNA strand. The oligonucleotides used for generating the mutants were so designed that at each mutation site, specific nucleotide changes were made to create desired codons for specific amino acid. This in vitro mutated double-stranded DNA was then transformed into competent E. coli TG1 cells, and phage plaques were screened by DNA sequencing to identify phages containing the correct mutation. The EcoRI DNA fragment encoding the mutated AdoHcy hydrolase gene was subcloned into the EcoRI site of pPROK-1 (Clontech, Palo Alto, CA) expression vector and transformed into competent E. coli JM109 cells. Transformed cells were selected against 100 μg/ml ampicillin. Ampicillin-resistant clones were inoculated into 20 ml of YT medium containing 35 μg/ml ampicillin for 2 h at 37°C, and then induced with 1 mM IPTG overnight. Cell-free extracts were analyzed by SDS-polyacrylamide gel electrophoresis to identify clones that inducibly overproduced a protein band at Mᵋ of about 45,000.

Overexpression and purification of mutant forms of AdoHcy hydrolase were carried out in the same conditions as that for the wild-type enzyme as described above.

Steady-state Kinetics—Kinetic constants were determined in both the synthetic and hydrolytic directions. The initial velocities for the enzyme reactions were measured using the methods as described in the enzyme activity assay section, except shorter reaction time (30 s) was used in the synthetic direction. For mutant enzymes of low activities, 4 to 10 times higher protein concentrations were used than that for the wild-type enzyme. Data were fitted to the Michaelis-Menten equation to obtain kcat and Kᵥ values using a nonlinear least squares fitting program (Ultrafit) run on a Macintosh computer. Changes in free energy (ΔG) for mutated enzymes were calculated using Equation 2 (37) (as follows).

\[ ΔG = -RT\ln \left( \frac{k_{\text{cat}}}{K_{\text{m,mutant}}} \right) - \ln(45,000) \]  

(Determination of E-NAD⁺ and E-NADH—Enzyme activity — and E-NADH contents of wild-type and mutant AdoHcy hydrolases were determined by an HPLC method as described previously (30).

Circular Dichroism Spectra—Circular dichroism (CD) spectra were recorded at 13°C using a Aviv-60DS spectropolarimeter (AVIV Associates Inc. Lakewood, NJ) equipped with a data processing system. Measurements were made with a cylindrical quartz cell with a path length of 0.1 cm. The concentration of the protein samples was 10 μM in buffer A. Five scans from 200 to 250 nm were recorded in 0.5-nm intervals for each sample. The scans for each sample were then averaged and corrected by subtracting a buffer base line. The relative percentages of α-helix, β-sheet, β-turn, and random coil structures were estimated using the AVIV version 3.1 computer program.

**RESULTS**

**Kinetics of AdoHcy Hydrolase Inactivation by DTNB**—Incubation of AdoHcy hydrolase with DTNB in 0.1 M phosphate buffer, pH 8.0, at 25°C resulted in a time-dependent loss of enzyme activity. The inactivation followed pseudo-first order kinetics (Fig. 1). After incubation for 50 min with 10 μM DTNB, about a 98% loss of enzyme activity was observed. The double logarithmic plot of the apparent first order rate constant (k_app) versus DTNB concentration is shown in the inset.

**Determination of the 3′-Reduction Rate from E-NAD⁺,3′-Keto-Ado to E-NAD⁺ and Ado**—The E-NAD⁺ form of the enzyme (E-NAD⁺) is reduced to the NADH form of the enzyme (E-NADH) when incubated with Ado. E-NAD⁺,2,8-[3H]3′-keto-Ado complex was prepared by incubation of the wild-type enzyme or mutant enzymes (1 mg in 1 ml of buffer A) with 200 μM [2,8-3H]-Ado (50 mCi/mmol) at 37°C for 15 min followed by passing through a Sephadex G-50 spin column to remove excess [2,8-3H]-Ado. The bound [2,8-3H]-Ado is oxidized to [2,8-3H]3′-keto-Ado concomitantly with the reduction of E-NAD⁺ to E-NADH based on the mechanism of the enzyme action (20) and our recent experimental observations that after denaturation of the wild-type enzyme incubated with Ado, the major reaction product released was 3′-keto-Ado (data not shown). The protein concentration and radioactivity of the E-NAD⁺,2,8-[3H]3′-keto-Ado complex were determined in order to calculate the stoichiometry of [2,8-3H]-Ado bound to AdoHcy hydrolase. The complex (~1 ml) was then dialyzed against a large volume (500 ml) of buffer A at 4°C. At various time intervals, two samples (30 μl each) were taken from the dialysis tubing to measure the protein concentration and radioactivity. The dialysis buffer was changed to new buffer A at each sampling time. The apparent rate constant for the 3′-reduction from E-NAD⁺,2,8-[3H]3′-keto-Ado to E-NAD⁺ and Ado was obtained by plotting the log of the percent [2,8-3H]-Ado bound versus the dialysis time, and the data were fitted to a linear least squares equation.

**Kinetics of AdoHcy Hydrolase Inactivation by DTNB**—Incubation of AdoHcy hydrolase with DTNB in 0.1 M phosphate buffer, pH 8.0, at 25°C resulted in a time-dependent loss of enzyme activity, and the inactivation followed pseudo-first order kinetics (Fig. 1). After incubation for 50 min with 10 μM DTNB, about a 98% loss of enzyme activity was observed. The double logarithmic plot of the apparent first order rate constant (k_app) versus DTNB concentrations yielded a straight line with a slope of 1.73, which represents the number of TNB bound per active site of the enzyme. When residual enzyme activity was plotted against the number of cysteine residues modified, it was shown that three cysteine residues per subunit were modified when the enzyme was completely inactivated (Fig. 2).
Modification and Mutation of Cysteines in AdoHcy Hydrolase

Modification and Mutation of Cysteines in AdoHcy Hydrolase

Cysteine Residues Modified per Enzyme Subunit

FIG. 2. Relationship between residual enzyme activity and number of cysteine residues modified by DTNB in the absence and presence of Ado. The wild-type enzyme (1.5 μM) was incubated with 10 μM DTNB in 0.1 M sodium phosphate buffer containing 1 mM EDTA, pH 8.0, at 25 °C. Aliquots were removed for assay of the enzyme activity at different times, while equivalents of cysteine residues modified were spectrophotometrically determined at 412 nm. C, in the absence of Ado; ●, in the presence of 10 μM Ado.

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The inactivation of AdoHcy hydrolase by DTNB was prevented in the presence of the substrate Ado or the competitive inhibitor Ade. Because of its tight binding with the enzyme, Ade was found to be an extremely strong protector against modification of the enzyme by DTNB. As shown in Fig. 2, in the presence of Ado (6.3-fold molar excess over the enzyme), only about one cysteine residue could be modified and the enzyme activity was almost completely protected when measured in the synthetic direction. This result indicated that the two cysteine residues protected by Ade may be located in or near the Ade binding site and could be essential for the enzyme activity. On the other hand, the cysteine residue that was not protected by Ade may be located outside of the Ade binding site and may not be essential for the enzyme activity. Enzyme inactivation by DTNB could also be prevented by Ade, although higher concentrations of Ade (500 μM) were required to achieve the same protective effect as that by Ado.

Double Inhibition Studies—Based on the observation that enzyme inactivation by DTNB could be prevented by Ade or Ado, double inhibition studies were performed to demonstrate that [1-14C]iodoacetamide modifies the same thiol groups as Ade, double inhibition studies were performed to demonstrate enzyme inactivation by DTNB could be prevented by Ado or protective effect as that by Ado.

Different forms of the wild-type enzyme (11.1 μM, native enzyme, ○, DTNB-modified Ade-protected enzyme; □, DTNB-modified enzyme without protection) were incubated with 2 mM [1-14C]iodoacetamide in 0.1 M Tris-HCl buffer, pH 8.9, at 25 °C in the dark for different times. At the indicated times, aliquots (50 and 3 μl) of the reaction mixture were removed for determination of stoichiometry of covalent binding and activity remaining. For stoichiometry of covalent binding, the modified enzyme was passed through a spin column (Sephadex G-50, 3 ml) to remove free [1-14C]iodoacetamide. The protein was then denatured with 8 M urea and passed through another spin column equilibrated with 1 M urea to remove any noncovalently bound [1-14C]iodoacetamide. The radioactivity and protein concentration were determined as described under the “Experimental Procedures.” For the remaining activity assay, the modified enzyme was mixed with 500 μl of buffer A containing 100 μM Ado and 5 mM Hcy as described previously (21), ▼, remaining activity of the native enzyme; ●, remaining activity of DTNB-modified Ade-protected enzyme.

The HPLC-purified radiolabeled peptides (approximately 50 pmol of each) were collected and rechromatographed on the same column with different buffer conditions. As shown in Fig. 4a, insets I and II, fractions a and b contained one major and several minor components with the radioactivity associated only with the major component (fractions a’ and b’). Similarly, one major fraction, which accounts for about 70% of the radioactivity, was observed from the tryptic digested enzyme modified for the cysteine residue located outside of the Ade binding site (Fig. 4b). Rechromatography of this major fraction generated several components, but only a single peak contained radioactivity (fraction c’) (Fig. 4b, inset).

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Isolation and Characterization of [1-14C]Iodoacetamide-modified Peptide Fragments of Tryptic Digestion—The enzymes that had been specifically modified with [1-14C]iodoacetamide for the two cysteines located in Ado binding site and the one located outside of the Ade binding site were subjected to tryptic digestion. Radiolabeled peptides were separated by reverse-phase HPLC. Fig. 4a shows the HPLC chromatogram from the enzyme specifically modified for the two cysteine residues in the Ado binding site. About 60% of the radioactivity was recovered from two major fractions (a and b), which were in an approximately 1:1 ratio. These two radiolabeled fractions were collected and rechromatographed on the same column with different buffer conditions. As shown in Fig. 4a, insets I and II, fractions a and b contained one major and several minor components with the radioactivity associated only with the major component (fractions a’ and b’). Similarly, one major fraction, which accounts for about 70% of the radioactivity, was observed from the tryptic digested enzyme modified for the cysteine residue located outside of the Ade binding site (Fig. 4b). Rechromatography of this major fraction generated several components, but only a single peak contained radioactivity (fraction c’) (Fig. 4b, inset).

Site-directed Mutagenesis—Cysteine residues (Cys113,
Cys<sup>195</sup>, and Cys<sup>421</sup>) identified by chemical modification were mutated to both serine (structurally conservative to cysteine) and aspartic acid (introducing a negative charge as DTNB does). All six mutants (C113S, C113D, C195S, C195D, C421S, and C421D) were overexpressed and purified by the same procedures used for the wild-type enzyme. No significant differences were observed in the levels of mutant enzyme expression or in the amount of mutant enzymes recovered in the purification system (data not shown). E-NAD<sup>+</sup> and E-NADH contents of mutant enzymes were similar to those of the wild-type enzyme, having approximately 0.8 mol of NAD<sup>+</sup> and 0.2 mol of NADH/mol of enzyme subunit except for C195D, which had less than a stoichiometric amount (0.6 mol of NAD<sup>+</sup> and 0.1 mol of NADH/mol of subunit).

**Steady-state Kinetic Properties of the Mutant Enzymes**—Table II shows kinetic parameters of mutant and wild-type enzymes and changes in binding energy (ΔΔG) of mutant enzymes. Michaelis-Menten constants were determined toward all three variable substrates: AdoHcy, Ado, and Hcy. Both Cys<sup>113</sup> mutants and Cys<sup>421</sup> mutants had only slightly increased $K_m$ values and slightly decreased $k_{cat}$ values toward all the three substrates compared with the $K_m$ and $k_{cat}$ values of the wild-type enzyme, indicating that mutating either Cys<sup>113</sup> or Cys<sup>421</sup> does not provoke any major changes in ground state binding and subsequent catalytic steps. In contrast, mutation of Cys<sup>195</sup> to either serine or aspartic acid led to large reductions in turnover numbers toward all three substrates. The $k_{cat}$ values of the C195D and C195S were 14- and 9-fold lower than those of the wild-type enzyme, which results in a calculated loss in binding energy (ΔΔG) of approximately 0.7–1.4 Kcal/mol (Table II). This indicates that the drastic losses of overall catalytic activities (93% loss for C195D and 88% loss for C195S) of the Cys<sup>195</sup> mutants are due mainly to effects on catalytic steps. The low $k_{cat}$ values of the Cys<sup>195</sup> mutants may have contributed to their relatively lower $K_m$ values. In addition, these losses of enzyme activity of the Cys<sup>195</sup> mutants were apparently not correlated to changes in the secondary structures of the enzymes, since there were no significant differences in the secondary structures between the mutants and the wild-type enzyme based on CD analysis (wild-type, $a$-helix = 23.4%, $b$-sheet = 16.7%, $b$-turn = 32.6%, random coils = 27.3%; C195S, $a$-helix = 23.4%, $b$-sheet = 16.6%, $b$-turn = 33.3%,

![Image](28013.png)
Effects of the Cys<sup>195</sup> Mutations on the Rate of 3'-oxidative and 5'-Hydrolytic Activities—In the hydrolytic direction, the overall catalytic activity of AdoHcy hydrolase requires two major sequential steps of catalysis: the 3'-oxidative activity and the 5'-hydrolytic activity. The 3'-oxidative activity can be measured by determining the rate of E-NAD<sup>+</sup> to E-NADH conversion, and the 5'-hydrolytic activity can be measured independently from the 3'-oxidation by determining the rate of hydrolytic product formation using EDDClHHA as a substrate (23). As seen in Fig. 5, both C195D and C195S showed slightly faster 3'-oxidation rates than that of the wild-type enzyme with apparent k<sub>a</sub> values of 0.11, 0.13, and 0.15 min<sup>-1</sup> for the wild-type, C195S, and C195D, respectively. However, due to the protection from the largely formed 5'-hydrolytic products (DCIHHA and Ade) (23), the conversion of E-NAD<sup>+</sup> to E-NADH by EDDClHHA was not complete as shown in Fig. 5.

The 5'-hydrolytic activities of the Cys<sup>195</sup> mutants and the wild-type enzyme were also shown to be similar, as seen in Fig. 6. From equation (1) and the apparent 3'-oxidation rate constants (k<sub>a</sub>)<sub>app</sub>, the apparent 5'-hydrolytic rate constants (k<sub>s</sub>) were calculated to be 0.72, 0.74, and 0.69 min<sup>-1</sup> for the wild-type, C195S, and C195D, respectively.

Effects of Cys<sup>195</sup> Mutations on the Rate of 3'-Reduction Reaction—Since the 3'-oxidative and 5'-hydrolytic reactions appeared not to be responsible for the severe loss of enzyme activity of the Cys<sup>195</sup> mutants, we focused our attention on the 3'-reduction step, which is the last chemical reaction step in both the synthetic and hydrolytic directions. The 3'-reduction reaction converts the tightly bound intermediates 3'-keto-Ado or 3'-keto-AdoHcy to the final reaction products Ado or AdoHcy concomitantly with the oxidation of E-NADH to E-NAD<sup>+</sup> to regenerate the active enzyme (NAD<sup>+</sup> form) for the next catalytic cycle. Comparison of the 3'-reduction rates between the Cys<sup>195</sup> mutants and the wild-type enzyme was accomplished by monitoring the rates of Ado release from the E-NADH, 3'-keto-

### Table II

| Enzyme | K<sub>m</sub> | k<sub>a</sub> | k<sub>cat</sub> | ΔΔG (K<sub>m</sub>,<sub>k</sub><sub>a</sub>)<sub>mutant</sub> | ΔΔG (K<sub>m</sub>,<sub>k</sub><sub>a</sub>)<sub>wild-type</sub> |
|--------|--------------|--------------|-------------|---------------------------------|---------------------------------|
| **Substrate: AdoHcy** | | | | | |
| Wild-type | 7.9 | 3.8 | 0.48 | 32.6% | 32.6% |
| C113S | 12.0 | 3.3 | 0.28 | 33.3% | 33.3% |
| C113D | 10.5 | 2.8 | 0.27 | 34.0% | 34.0% |
| C113D | 2.7 | 0.44 | 0.16 | 66.7% | 66.7% |
| C195D | 4.1 | 0.27 | 0.07 | 64.3% | 64.3% |
| C241S | 10.6 | 3.8 | 0.36 | 34.0% | 34.0% |
| C241D | 11.8 | 3.1 | 0.26 | 37.8% | 37.8% |
| **Substrate: Ado** | | | | | |
| Wild-type | 1.4 | 9.7 | 6.9 | 35.2% | 35.2% |
| C113S | 1.9 | 9.2 | 4.9 | 20.0% | 20.0% |
| C113D | 1.8 | 9.1 | 5.1 | 19.0% | 19.0% |
| C195S | 1.2 | 1.1 | 0.91 | 12.0% | 12.0% |
| C195D | 1.0 | 0.69 | 0.69 | 10.0% | 10.0% |
| C241S | 1.6 | 9.5 | 5.9 | 19.0% | 19.0% |
| C241D | 1.9 | 9.1 | 4.8 | 22.0% | 22.0% |
| **Substrate: Hcy** | | | | | |
| Wild-type | 88.1 | 9.8 | 0.11 | 26.7% | 26.7% |
| C113S | 92.3 | 9.3 | 0.10 | 26.7% | 26.7% |
| C113D | 99.7 | 9.2 | 0.09 | 26.7% | 26.7% |
| C195S | 78.8 | 1.2 | 0.15 | 18.7% | 18.7% |
| C195D | 65.3 | 0.67 | 0.10 | 14.1% | 14.1% |
| C241S | 83.6 | 9.1 | 0.10 | 26.7% | 26.7% |
| C241D | 94.9 | 8.9 | 0.093 | 10.0% | 10.0% |

random coils = 26.7%; C195D, α-helix = 25.0%, β-sheet = 12.6%, β-turn = 35.2%, random coils = 27.2%).

Effects of the Cys<sup>195</sup> Mutations on the 3'-Oxidative and 5'-Hydrolytic Activities—In the hydrolytic direction, the overall catalytic activity of AdoHcy hydrolase requires two major sequential steps of catalysis: the 3'-oxidative activity and the 5'-hydrolytic activity. The 3'-oxidative activity can be measured by determining the rate of E-NAD<sup>+</sup> to E-NADH conversion, and the 5'-hydrolytic activity can be measured independently from the 3'-oxidation by determining the rate of hydrolytic product formation using EDDClHHA as a substrate (23). As seen in Fig. 5, both C195D and C195S showed slightly faster 3'-oxidation rates than that of the wild-type enzyme with apparent k<sub>a</sub> values of 0.11, 0.13, and 0.15 min<sup>-1</sup> for the wild-type, C195S, and C195D, respectively. However, due to the protection from the largely formed 5'-hydrolytic products (DCIHHA and Ade) (23), the conversion of E-NAD<sup>+</sup> to E-NADH by EDDClHHA was not complete as shown in Fig. 5.

The 5'-hydrolytic activities of the Cys<sup>195</sup> mutants and the wild-type enzyme were also shown to be similar, as seen in Fig. 6. From equation (1) and the apparent 3'-oxidation rate constants (k<sub>a</sub>)<sub>app</sub>, the apparent 5'-hydrolytic rate constants (k<sub>s</sub>) were calculated to be 0.72, 0.74, and 0.69 min<sup>-1</sup> for the wild-type, C195S, and C195D, respectively.

Effects of Cys<sup>195</sup> Mutations on the Rate of 3'-Reduction Reaction—Since the 3'-oxidative and 5'-hydrolytic reactions appeared not to be responsible for the severe loss of enzyme activity of the Cys<sup>195</sup> mutants, we focused our attention on the 3'-reduction step, which is the last chemical reaction step in both the synthetic and hydrolytic directions. The 3'-reduction reaction converts the tightly bound intermediates 3'-keto-Ado or 3'-keto-AdoHcy to the final reaction products Ado or AdoHcy concomitantly with the oxidation of E-NADH to E-NAD<sup>+</sup> to regenerate the active enzyme (NAD<sup>+</sup> form) for the next catalytic cycle. Comparison of the 3'-reduction rates between the Cys<sup>195</sup> mutants and the wild-type enzyme was accomplished by monitoring the rates of Ado release from the E-NADH, 3'-keto-
Ado complex in a dialysis system. As the dissociation of Ado from $E^{-NAD^+}$:Ado is a fast step with a rate constant of 2 s$^{-1}$ for wild-type enzyme (38), the measurement of the rate of Ado release from $E^{-NADH}$:3'-keto-Ado complex is essentially a measure-
ment of the rate of the 3'-reduction (rate-determining step). As shown in Fig. 7, the apparent 3'-reduction rate constants for C195S and C195D were found to be $3.2 \times 10^{-6}$ s$^{-1}$ and $2.5 \times 10^{-6}$ s$^{-1}$, respectively, which were 5.1- and 6.5-fold slower than that of the wild-type enzyme ($16.3 \times 10^{-6}$ s$^{-1}$). It should be noted that the values of the apparent 3'-reduction rate con-
stants obtained in this system were not the true values of the effective 3'-reduction rate constants for the enzymes during catalysis, since there was no substrate competition in the dial-
ysis system. However, these apparent rate constants serve the purpose for comparison of the 3'-reduction rates between the mutants and the wild-type enzyme under the same conditions.

**DISCUSSION**

Due to lack of an x-ray crystal structure of AdoHcy hydrolase, little is known about the amino acid residues involved in the substrate binding and/or catalysis of the enzyme. In this study, we have utilized the thiol-specific reagents DTNB and iodoacacetamide to probe the active site of AdoHcy hydrolase and have employed site-directed mutagenesis to characterize the cysteine mutants identified by chemical modification. From these studies, it is concluded that Cys$^{195}$ is important for the enzyme to exert its full catalytic activity.

There are 10 cysteine residues per subunit of human placen-
tal AdoHcy hydrolase. Incubation of the enzyme with DTNB or iodoacacetamide resulted in complete loss of enzyme activity when three cysteine residues (Cys$^{113}$, Cys$^{195}$, and Cys$^{421}$) were modified. Kinetic studies on enzyme inactivation by DTNB showed that the loss of enzyme activity was due to modification of two (Cys$^{113}$ and Cys$^{195}$) of the three modifiable cysteine residues. Protection of these two cysteine residues with the substrate Ado or the competitive inhibitor Ade led to protection of the enzyme from inactivation, suggesting that Cys$^{113}$ and Cys$^{195}$ may be located in or near the enzyme active site. These results are consistent with earlier results, which indicated that Cys$^{113}$ in the rat liver AdoHcy hydrolase (29), comparable with the Cys$^{113}$ in human enzyme, and Glu$^{197}$ in human AdoHcy hydrolase (31), only two residues away from the Cys$^{195}$, are located in or near the active site of AdoHcy hydrolase. Modification of Cys$^{421}$ alone did not effect the enzyme activity, indicating that Cys$^{421}$ is a nonessential residue; this is also sup-
ported by the site-directed mutagenesis results, since mutations of this residue with serine and aspartic acid had little effects on the enzyme activity, which is consistent with mutational observations of Cys$^{113}$ in the rat AdoHcy hydrolase (32).

In contrast, mutations of the Cys$^{195}$ with serine or aspartic acid resulted in drastic loss of enzyme activity. This loss of activity is concluded to be mainly due to the perturbation of the 3'-reduction potential necessary for efficient release of the reaction product and regeneration of the active form (NAD$^+$ form) of the enzyme. Direct evidence that supports this conclusion is that the rates of Ado release from the $E^{-NADH}$:3'-keto-
Ado complex of the Cys$^{195}$ mutants are 5-6.5-fold slower than that of the wild-type enzyme. It seems that the decrease in the 3'-reduction rate alone does not account for the overall losses of the enzyme activities (9-14-fold), especially for the C195D mutant. However, when the NAD$^+$ content of the C195D mutant, which is 30% less than that of the wild-type enzyme, is taken into account, then the loss of enzyme activity of the C195D mutant is only 9.8-fold, which is quite close to its loss of the 3'-reduction rate. Other evidence that indirectly supports the conclusion includes: (i) the Cys$^{195}$ mutants have slightly higher 3'-oxidation activities and similar 5'-hydrolytic activi-
ties compared with the wild-type enzyme, suggesting that the loss of enzyme activity of the Cys$^{195}$ mutants is not caused by dysfunctions of these two major catalytic steps, and (ii) the secondary structures of the Cys$^{195}$ mutant and the wild-type enzyme are basically the same, indicating that the loss of enzyme activity of the Cys$^{195}$ mutant is not correlated to alternations in secondary structures. Exclusion of these two inactiva-
tion possibilities led us to focusing on the 3'-reduction reaction. The Cys$^{195}$ residue may be involved in maintaining the 3'-reduction potential by forming a hydrogen bond with the 3'-C=O or 3'-OH of the ribose ring of 3'-keto-Ado or Ado, respectively. It has been reported that, based on an x-ray crystal structure, the SH group of Cys$^{195}$ in tyrosyl-tRNA synthetase forms a hydrogen bond with the 3'-OH of the ribose ring of ATP (37). Mutation of this Cys$^{195}$ to serine results in significant loss of enzyme activity and a calculated loss of free energy (ΔG$^*$) of approximately 1 Kcal/mol, which is equal to the value of the binding energy of the hydrogen bond between the SH group and the 3'-OH of ATP (37). Similar binding energy losses (1.2 Kcal/mol) were observed for the Cys$^{195}$ mutants, indicating that mutation of Cys$^{195}$ may lead to a loss of a hydrogen bond between the SH group and the 3'-C=O of the ribose ring of 3'-keto-Ado. It is not surprising that loss of this hydrogen bond impairs the 3'-reduction reaction, since hydrogen bond formation between the SH group and the 3'-C=O may help to facilitate the electron or hydride transfer from the pyridine
ring of NADH to the 3'-carbonyl carbon. If this is the case, then the reverse reaction, i.e. the 3'-oxidation reaction, would be enhanced as the loss of the hydrogen bond between the —SH group and the 3'-OH would benefit the electron or hydride transfer from the 3'-OH and 3'-CH to the pyridine ring of NAD+. This is also consistent with the observation that the 3 '-oxidation rate constants of the Cys195 mutants are slightly larger than that of the wild-type enzyme.

If the hydrogen bond between the —SH group of the Cys195 and the 3'-C=O is critical to the 3'-reduction potential, it might be expected that a conservative mutation of Cys195 to serine would have no significant effect on enzyme activity, since the —OH group of the serine can also form a hydrogen bond with the 3'-C=O of the ribose ring of 3'-keto-Ado. However, the optimum OH—O hydrogen bond distance is at least 0.4 Å shorter than the corresponding SH—O distance (13, 14). Thus, since the —SH of the Cys195 is in a position to make the optimal hydrogen bond with the 3'-C=O of the ribose ring of 3'-keto-Ado, a hydrogen bond between —OH of the serine and the 3'-C=O would be at least 0.5 Å longer than the optimum, which would contribute little to the binding energy and thus to the 3'-reduction potential.

In summary, this study has identified three DTNB modifiable cysteine residues per subunit of human AdoHcy hydrolase. The Cys421 residue is nonessential and is not located in the Ado binding site. Identification of this nonessential cysteine residue has been found to be useful in molecular probing and derivatization of the enzyme. The Cys113 residue is located in or near the Ado binding site, but plays no significant role beneficial to the enzyme binding or catalysis. The Cys195 residue is involved in the catalytic center and is critical to the full catalytic function of the enzyme. Cys195 is most likely involved in the 3'-reduction step in the overall catalytic pathway and may play a role in maintaining the 3'-reduction potential by hydrogen bond formation with the 3'-C=O of the ribose of 3'-keto-Ado. However, Cys195 is not absolutely required for the enzyme activity, as demonstrated by the residual enzyme activities of the Cys195 mutants and the evolutional mutation of Cys195 that is replaced by isoleucine in nematode Caenorhabditis elegans (18), although it is conserved in all nine of the other known AdoHcy hydrolases from highly evolutionally divergent species (11).

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