Evidence for an Essential Histidine Residue in *S*-Adenosylhomocysteinase from Rat Liver†

Tomoharu Gomi and Motoji Fujioka*

ABSTRACT: Rat liver *S*-adenosylhomocysteinase (EC 3.3.1.1) is inactivated by diethyl pyrocarbonate. The inactivation is first order in enzyme and in reagent, and a second-order rate constant of 77 M^{-1} min^{-1} is obtained at pH 6.9 and 0 °C. The rate of inactivation is dependent on pH, and the pH-inactivation rate data show the involvement of a group with a pK of 6.8. The difference spectrum of the inactivated and native enzymes shows a single peak at 242 nm, indicating the modification of histidine residues. No trough at around 280 nm due to *O*-carbethoxytyrosine is observed. The sulfhydryl content of the enzyme is unchanged by the reaction. The inactivation was reversed by hydroxylamine. Although the reaction with [^{3}H]diethyl pyrocarbonate reveals that a residue(s) other than histidine is (are) also modified, the agreement of the number of histidine residues modified and the number of carbethoxy groups removed by hydroxylamine treatment indicates that the inactivation is solely due to the modification of histidine. Statistical analysis of the residual enzyme activity and the extent of modification shows that, among six modifiable residues per subunit, one which reacts more rapidly with the reagent than the rest is critical for activity. The modified enzyme still retains the capacity to bind adenosine and *S*-adenosylhomocysteine and to oxidize the 3′-hydroxyl of these compounds as evidenced by the reduction of the enzyme-bound NAD^+. Slow but significant exchange of the 4′ proton with solvent also occurs with the modified enzyme. Thus, it may be concluded that the histidine residue essential for activity is involved in a catalytic reaction other than the abstraction of 3′-hydroxyl and 4′ protons of the substrates.

S-Adenosylhomocysteinase (EC 3.3.1.1) catalyzes the reversible cleavage of the thioether bond of *S*-adenosyl-L-homocysteine to yield adenosine and L-homocysteine. *S*-Adenosylhomocysteinase of rat liver is a tetramer consisting of apparently identical subunits. The enzyme monomer contains 1 mol of tightly bound NAD^+ as the coenzyme and possesses one binding site for substrates (Fujioka & Takata, 1981). The enzyme shows no cooperativity in the binding of adenosine or in kinetics.

The chemical mechanism of the reaction catalyzed by the enzyme has been studied in detail by Palmer & Abeles (1979). The substrate adenosine or *S*-adenosylhomocysteine is first oxidized to a 3′-keto derivative by NAD^+, which then undergoes proton abstraction from the 4′ position. The resulting α-ketocarbanion eliminates water or homocysteine to form 3′-keto-4′,5′-dehydroadenosine. Addition of homocysteine or water to this central intermediate and the reversal of the pathway result in the formation of product. To explore the possibility that histidine residue(s) of the enzyme is (are) involved in the removal or addition of protons, we have carried out a chemical modification study using a histidine-selective reagent diethyl pyrocarbonate. The results reported herein show that diethyl pyrocarbonate inactivates *S*-adenosylhomocysteinase by modifying one histidine residue per subunit, but the carbethoxylation does not prevent the abstraction of protons from the 3′ and 4′ positions of substrates.

Experimental Procedures

Materials. *S*-Adenosyl-L-homocysteine, adenosine, DL-homocysteine thiolactone, calf intestinal mucosa adenosine deaminase (EC 3.5.4.4) (type III), and yeast alcohol dehydrogenase (EC 1.1.1.1) were obtained from Sigma Chemical Co. Diethyl pyrocarbonate was a product of Aldrich Chemical Co. [2,8-$^3H_2$]Adenosine (34.4 Ci/mmol) and [1-^{3}H]ethanol (100 mCi/mmol) were purchased from New England Nuclear, and tritiated water (5 Ci/mL) was from Amersham. DL-Homocysteine was prepared from the thiolactone by treatment with 2 N NaOH for 10 min at room temperature followed by neutralization with KH_2PO_4 as described by Duerre & Miller (1966) and stored under nitrogen. Other chemicals were of the highest grade available from commercial sources. *S*-Adenosylhomocysteinase was purified to homogeneity from rat liver by the method of Fujioka & Takata (1981). The experiments described below were carried out with the homogeneous enzyme preparation.

Synthesis of [^3H]Diethyl Pyrocarbonate. Tritiated diethyl pyrocarbonate was synthesized from [1-^{3}H]ethanol as described by Melchior & Fahrney (1970). The product had a specific activity of 41.1 μCi/mmol. Since the molecule is symmetrical and the radioactive product is labeled only in one position, the specific activity of a carbethoxylated residue was assumed to be 20.6 μCi/mmol.

Reaction of S-Adenosylhomocysteinase with Diethyl Pyrocarbonate. Diethyl pyrocarbonate was freshly diluted with cold ethanol for each experiment. Since the concentrations of commercial products are variable owing to hydrolysis, the concentration of the reagent was determined on each diluted sample by the reaction with imidazole. An aliquot of the dilution was added to 10 mM imidazole at pH 7.5, and the concentration was calculated from the increase in absorbance at 230 nm due to the formation of *N*-carbethoxyimidazole, using a molar absorptivity of 3×10^3 M^{-1} cm^{-1} (Melchior & Fahrney, 1970). Carboethoxylation was carried out by incubating *S*-adenosylhomocysteinase with an appropriately diluted reagent in 0.1 M potassium phosphate buffer. The final concentration of ethanol in reaction mixtures never exceeded 5%. The extent of inactivation was determined by measuring the residual enzyme activity at 25 °C on an aliquot removed from the reaction mixture as described below. The enzyme activity was determined in both the synthetic and hydrolytic directions of *S*-adenosylhomocysteine. The extent of inactivation was precisely the same in both assays. Unless otherwise

† From the Department of Biochemistry, Toyama Medical and Pharmaceutical University Faculty of Medicine, Sugitani, Toyama 930-01, Japan. *Received June 2, 1982.* This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

0006-2960/83/0422-0137$01.50/0

indicated, the activity measurements were made in the direction of hydrolysis.

Enzyme Assays. The *S*-adenosylhomocysteinase activity in the direction of synthesis was determined by the rate of formation of [^{3}H]adenosylhomocysteine from [^{3}H]adenosine and homocysteine. The standard assay mixture contained 20 μM [2,8-$^3H_2$]adenosine (20 K_m) and 5.0 mM DL-homocysteine (32 K_m) in 50 μL of 20 mM potassium phosphate buffer, pH 7.2. The reaction was terminated by the addition of 5 μL of 10% trichloroacetic acid, and the [^{3}H]adenosylhomocysteine formed was separated from [^{3}H]adenosine by chromatography on DEAE-cellulose paper (Whatman DE 81) and its radioactivity determined (Fujioka & Takata, 1981). The assay in the hydrolysis direction was performed in an assay mixture containing 50 μM *S*-adenosylhomocysteine (3.7 K_m) and 1.0 IU of adenosine deaminase in 2.0 mL of 20 mM potassium phosphate buffer, pH 6.9. The decrease in absorbance due to the conversion of the product adenosine to inosine was followed spectrophotometrically at 265 nm (Fujioka & Takata, 1981). This spectrophotometric assay is rather sensitive ($\Delta\epsilon = 8.1 \times 10^3$ M^{-1} cm^{-1}) and is linear with time within the absorbance change of at least 0.05. Under these conditions, the diethyl pyrocarbonate carried over to the assay mixture (<30 μM) did not interfere with the assay.

Determination of Enzyme-Bound NAD$^+$ and NADH. The enzyme-bound NAD$^+$ and NADH formed by the reaction of the enzyme with adenosine were determined on a perchloric acid extract and ethanol/KOH extract of the enzyme, respectively. The procedures for determinations are described elsewhere (Fujioka & Takata, 1981; Gomi & Fujioka, 1982).

Determination of Total Sulfhydryl Residues. The determination of total sulfhydryl residues after diethyl pyrocarbonate inactivation was made by the method of Ellman (1959). After separation of excess reagent by gel filtration on a Sephadex G-25 column (1.8 × 12 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, the sulfhydryl content was determined with 5,5′-dithiobis(2-nitrobenzoate) in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.2% sodium dodecyl sulfate and 0.45 M $(NH_4)_2SO_4$.

Equilibrium Dialysis. The binding of adenosine to the diethyl pyrocarbonate inactivated enzyme was determined by the equilibrium dialysis method. The equilibrium dialysis was carried out in 20 mM potassium phosphate buffer, pH 7.2, with [2,8-$^3H_2$]adenosine as ligand (Gomi & Fujioka, 1982).

Synthesis of [4′-^{3}H]Adenosine and Assay of 4′ Proton Exchange Activity. [4′-^{3}H]Adenosine was prepared by incubating adenosine with *S*-adenosylhomocysteinase in [^{3}H]-H_2O as described by Palmer & Abeles (1979) and purified by chromatography on DEAE-cellulose paper (Gomi & Fujioka, 1982).

The exchange of the 4′ proton of adenosine with solvent was determined as follows. The enzyme was incubated with 0.39 mM [4′-^{3}H]adenosine (490 K_d; 2.7 × 10^5 dpm/μmol) in 0.2 mL of 20 mM potassium phosphate buffer, pH 7.2. At the appropriate time, the reaction was terminated by adding 10 μL of 1.0 M $HClO_4$ (0 °C). The mixture was then applied to a column (0.55 × 2.5 cm) of Dowex 50-X8, H$^+$ form, and the column was washed with 3 mL of water. The radioactivity of the eluate was determined in a scintillant containing Triton X-100.

Other Analytical Procedures. The protein concentration was determined by the method of Lowry et al. (1951). A subunit molecular weight of 47 000 for *S*-adenosylhomocysteinase (Fujioka & Takata, 1981) was used in all calculations. Spectrophotometric analyses were made in a Hitachi Model 320 recording spectrophotometer. Fluorescence measurements were carried out with a Farrand spectrofluorometer MK-2 and radioactivity measurements with an Aloka liquid scintillation spectrometer, Model LSC 903.

Results

Inactivation of S-Adenosylhomocysteinase by Diethyl Pyrocarbonate. Incubation of *S*-adenosylhomocysteinase with diethyl pyrocarbonate in 0.1 M potassium phosphate buffer resulted in a time-dependent loss of enzyme activity. After incubation for 30 min with 3 mM diethyl pyrocarbonate at pH 6.9 and 0 °C, less than 1% of the original activity was observed as measured by the standard assay. The low temperature was chosen to decrease the rate of hydrolysis of the reagent. Despite the presence of the reagent in great molar excess over the enzyme, a semilog plot of residual enzyme activity vs. time did not yield a straight line; the rate of inactivation decreased with time (not shown). This is apparently due to the instability of diethyl pyrocarbonate. Diethyl pyrocarbonate is readily hydrolyzed in aqueous solutions, the half-life being dependent on temperature, pH, and the concentration and composition of the buffer (Berger, 1975). Thus, when the decomposition of the reagent is taken into account and under the assumption that the modified enzyme is totally inactive, the fraction of enzyme activity remaining at time t (A/A_0) may be expressed as (the decrease in concentration of the reagent due to the reaction with the enzyme being neglected)

$$\ln (A/A_0) = -(k/k')I_0(1 - e^{-k't}) \quad (1)$$

where I_0 is the initial concentration of diethyl pyrocarbonate, k is the bimolecular rate constant for reaction of the enzyme with the reagent, and k' is the first-order rate constant for hydrolysis of the reagent. Therefore, a plot of ln (A/A_0) against $(1 - e^{-k't})/k'$ should be linear. The value of k' was determined by assaying the diethyl pyrocarbonate concentration in the reaction mixture. At time intervals, aliquots were removed, and the concentration of diethyl pyrocarbonate was determined on each aliquot by the reaction with imidazole as described under Experimental Procedures. In 0.1 M potassium phosphate buffer, pH 6.9, the value of k' was found to be 7.5 × 10^{-3} min^{-1} at 0 °C. With this value of k', when a plot was made of log (A/A_0) vs. $(1 - e^{-k't})/k'$, a straight line was indeed obtained (Figure 1A), showing that the assumptions made above are valid. The slopes of plots (kI_0) obtained at different concentrations of diethyl pyrocarbonate were linearly related to the concentrations of the reagent (Figure 1B), indicating that no reversible complex is formed prior to the inactivation process. The second-order rate constant for inactivation (k) was calculated to be 77 M^{-1} min^{-1} from the data of Figure 1B.

Effect of Modification on Steady-State Kinetics. To test whether the loss of activity after modification is due to the change in V_{max} or in K_m for substrate, we examined the effect of substrate concentration on the reaction velocity with a partially inactivated enzyme preparation in the direction of *S*-adenosylhomocysteine hydrolysis. If an enzyme species with increased K_m is present, the double-reciprocal plot of initial velocities vs. substrate concentrations would show a curvature which is concave down near the vertical axis. To detect a possible deviation of the double-reciprocal plot from linearity, we have used an enzyme preparation that had been inactivated to as low as 31% of the original activity as measured by the standard assay (*S*-adenosylhomocysteine, 50 μM). As shown in Figure 2, the plot was a straight line with an identical K_m value with that of the unmodified enzyme (13.5 μM). Under

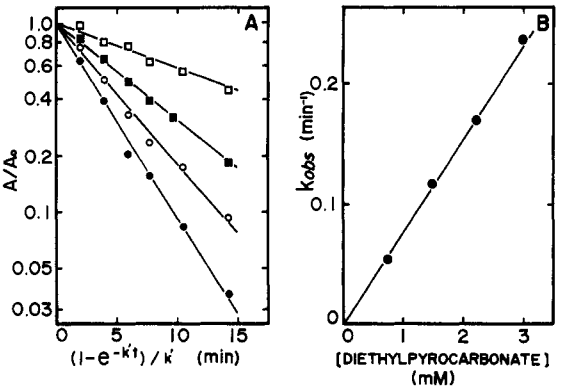

FIGURE 1: Inactivation of *S*-adenosylhomocysteinase by diethyl pyrocarbonate. (A) The enzyme (16 μM subunit) was incubated with 0.74 (□), 1.48 (■), 2.21 (○), or 2.98 mM (●) diethyl pyrocarbonate in 0.1 M potassium phosphate buffer, pH 6.9, at 0 °C. At time intervals, aliquots were removed for measurements of the residual enzyme activity. The first-order rate constant for decomposition of diethyl pyrocarbonate (k') was determined separately as described in the text. Values on the abscissa are calculated with $k' = 7.5 \times 10^{-3}$ min^{-1}. *S*-Adenosylhomocysteinase incubated in the absence of diethyl pyrocarbonate lost no activity under the conditions. (B) Plot of apparent first-order rate constants for inactivation (k_{obsd}) obtained at various concentrations of diethyl pyrocarbonate against concentrations of the reagent.

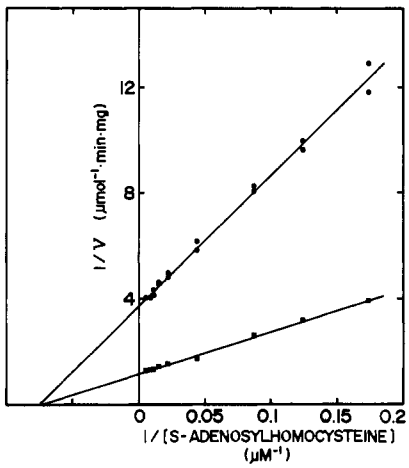

FIGURE 2: Double-reciprocal plots of initial velocities against *S*-adenosylhomocysteine concentrations. The rate measurement was made at 25 °C in a reaction mixture containing *S*-adenosylhomocysteine, 1.0 IU of adenosine deaminase, and 8.5 μg of the native enzyme (■) or 35.8 μg of the partially inactivated enzyme (●) in 2.0 mL of 20 mM potassium phosphate buffer, pH 6.9. The *S*-adenosylhomocysteine concentration was varied from 5.75 to 184 μM. The partially inactivated enzyme was prepared as follows. *S*-Adenosylhomocysteinase (2.2 mg) was incubated with 1.2 mM diethyl pyrocarbonate in 0.5 mL of 0.1 M potassium phosphate buffer, pH 6.9, at 0 °C. After incubation for 25 min, 0.5 mL of 40 mM imidazole (pH 7.6) was added to stop further inactivation, and the mixture was passed through a column of Sephadex G-25 (1.8 × 12 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.9. The specific activity of the modified enzyme was 31% of that of the native enzyme as determined under standard assay conditions. The lines were drawn by a least-squares linear regression.

the experimental conditions, even a 100-fold increase in K_m could be detected. If the residual activity measured under the standard assay conditions represents the sum of the activity of unmodified enzyme remaining and that of modified enzyme whose K_m is increased 100-fold but V_{max} unaltered, the velocities at 140 and 180 μM *S*-adenosylhomocysteine, for example, would be about 13 and 19% higher, respectively, than those obtained when the modified enzyme is totally inactive. The difference could be detected in view of the accuracy of the assay method used.

Table I: Comparison of Carbethoxyhistidines and [^{3}H]Carbethoxy Groups of Diethyl Pyrocarbonate Treated *S*-Adenosylhomocysteinase[a]

carbethoxyhistidine[b] (mol/mol of subunit)	[^{3}H]carbethoxy group (mol/mol of subunit)		
	before NH_2OH (A)	after NH_2OH (B)	A − B
0.66	0.81	0.12	0.69
1.18	1.59	0.23	1.36
1.99	2.95	0.47	2.48
4.08	4.69	1.12	3.57
4.57	6.00	1.07	4.93

[a] The enzyme (1.2 mg/mL) was incubated with 0.5 mM [^{3}H]-diethyl pyrocarbonate (9.04×10^4 dpm/μmol) in 0.1 M potassium phosphate buffer, pH 6.9, at 10 °C. At appropriate times, the absorbance at 242 nm was recorded, and an aliquot was removed and added with 1/9 volume of 0.2 M imidazole at pH 7.5 (25 °C) to stop further modification. The mixture was then passed over a column of Sephadex G-25 (1.2 × 18 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.9, and the void volume fraction was concentrated by ultrafiltration (Sartorius membrane SM 13200). A portion of the concentrated enzyme solution was measured for radioactivity, and the remainder was treated with 0.5 M hydroxylamine for 2 h at pH 7 and 25 °C. The radioactivity of the hydroxylamine-treated enzyme was measured after gel filtration as above. [b] Determined spectrophotometrically.

The experiment described below (Figure 6) shows that the modification results in an increase in K_d for adenosine from 0.8 to 14 μM. A modified enzyme for which no activity could be found by the standard assay (20 μM adenosine) failed to catalyze the synthesis of *S*-adenosylhomocysteine even when 500 μM adenosine was used. Thus, the results are consistent with the contention that the modification abolishes the catalytic turnover of the enzyme. In subsequent experiments, the residual activities after modification are determined by the standard assay. The absence of enzyme species with different catalytic activity would make it a valid measure of the amount of unmodified enzyme remaining.

Amino Acid Residues Modified by Diethyl Pyrocarbonate. The difference spectrum of the diethyl pyrocarbonate treated and native enzymes revealed a peak with an absorption maximum at 242 nm, characteristic of *N*-carbethoxyhistidine residue in protein (not shown) (Ovádi et al., 1967). Complete absence of spectral change above 270 nm rules out the modification of tyrosine residue. O-Carbethoxylation of tyrosine residue shows a negative difference spectrum at 278 nm (Mühlárd et al., 1967). The diethyl pyrocarbonate treatment did not result in appreciable change in the cysteine content. When an almost completely inactivated enzyme was examined for its sulfhydryl content with 5,5′-dithiobis(2-nitrobenzoate) as described under Experimental Procedures, a value of 7.63 residues per subunit was obtained. A parallel determination on the untreated enzyme showed that it contained 7.68 residues per subunit.

In addition to the residues mentioned above, diethyl pyrocarbonate is known to react with serine and arginine residues as well as α- and ϵ-amino groups (Mühlárd et al., 1967; Melchior & Fahrney, 1970; Wells, 1973; Burnstein et al., 1974). When *S*-adenosylhomocysteinase was incubated with [^{3}H]diethyl pyrocarbonate, incorporation of the radiolabel was observed concomitant with loss of enzyme activity. The number of carbethoxy groups incorporated as determined by radioactivity measurement exceeded that of histidine residues modified as determined from the difference absorbance at 242 nm [$\epsilon = 3.2 \times 10^3$ M^{-1} cm^{-1} (Setlow & Mansour, 1970; Burnstein et al., 1974)], showing that the reaction of diethyl

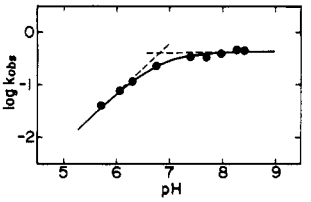

FIGURE 3: Effect of pH on inactivation. The enzyme (21.2 μM subunit) was incubated with 2.7 mM diethyl pyrocarbonate in 0.1 M potassium phosphate buffer at pH values indicated at 0 °C. The value of k' was determined at each pH, and the apparent first-order rate constant for inactivation was obtained as in Figure 1A. The *points* in the figure represent the experimental values, and the *curve* is calculated from the equation $k_{obsd} = k/[1 + ([H]/K_a)]$ with $k = 0.42$ min^{-1} (the pH-independent value) and $K_a = 10^{-6.8}$. pH measurements were made with 0.1 M buffer solutions at 0 °C.

pyrocarbonate also occurs at residues other than histidine (Table I). Treatment of the inactivated enzyme with 0.5 M hydroxylamine at pH 7 and 25 °C resulted in an almost complete recovery (>95%) of enzyme activity within 60 min. The hydroxylamine treatment also released most of the radiolabel. The number of carbethoxy groups removed was in good agreement with that of carbethoxyhistidines (Table I). The hydroxylamine-stable label was retained even after treatment for 12 h. Neutral hydroxylamine removes the carbethoxy groups from modified histidine, tyrosine, and serine, but not from lysine, arginine, cysteine, and the NH_2-terminal amino group (Melchior & Fahrney, 1970; Miles, 1977). Therefore, the results rule out the modification of tyrosine and serine residue and indicate that the inactivation of *S*-adenosylhomocysteinase by diethyl pyrocarbonate is solely due to the modification of histidine residue(s), even though modification of other residues is evident.

Effect of pH on Inactivation. The pH dependence of inactivation was studied in 0.1 M potassium phosphate buffer between pH 5.6 and 8.5. The rate of inactivation increased with increasing pH up to pH 7.5. The plot of apparent first-order rate constants for inactivation obtained at different pH vs. pH showed a typical titration curve with a pK of 6.8 (Figure 3). Since diethyl pyrocarbonate reacts only with the unprotonated form of imidazole in a model system and in protein (Holbrook & Ingram, 1973), and the pK value found is within the range expected for a histidine residue, the result is consistent with the proposal that the inactivation results from the modification of histidine residue(s).

Lack of Effect of Modification on NAD$^+$ Content, Molecular Size, and Fluorescence Property of Enzyme. Rat liver *S*-adenosylhomocysteinase contains 1 mol of tightly bound NAD$^+$/mol of subunit, which is not released by dialysis or gel filtration. Since the bound NAD$^+$ is essential for catalytic activity of the enzyme (Fujioka & Takata, 1981; Palmer & Abeles, 1976, 1979), the reversibility of inactivation by hydroxylamine (Table I) suggests that it is not released by the modification. This was confirmed by the direct measurement of NAD$^+$ content after modification. When the enzyme was inactivated to less than 1% of the original activity, the enzyme was isolated by gel filtration through a column of Sephadex G-25, and a perchloric acid extract thereof was prepared (Fujioka & Takata, 1981). Reaction of the neutralized extract with ethanol and alcohol dehydrogenase showed the formation of a compound which was indistinguishable spectrophotometrically from NADH. Calculation from the molar absorptivity at 340 nm (6.22×10^3 M^{-1} cm^{-1}) gave a value of 0.93 mol/mol of subunit.

When chromatographed on a Sephadex G-200 column, the diethyl pyrocarbonate inactivated enzyme was eluted as a

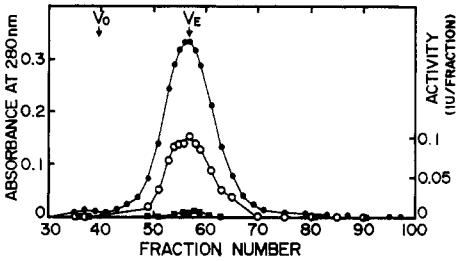

FIGURE 4: Sephadex G-200 column chromatography of the inactivated enzyme. The enzyme (0.11 mM subunit) was inactivated by treatment with 2 mM diethyl pyrocarbonate for 30 min at pH 6.9 and 0 °C. The inactivated enzyme was applied to a column (1.6 × 56 cm) of Sephadex G-200 previously equilibrated with 50 mM potassium phosphate buffer, pH 7.2, and eluted with the same buffer at a flow rate of 10 mL/h. Absorbance at 280 nm (●); enzyme activity before (■) and after (○) treatment with 0.5 M hydroxylamine. The *arrows* indicate the void volume (V_0) and the elution position of the native enzyme (V_E).

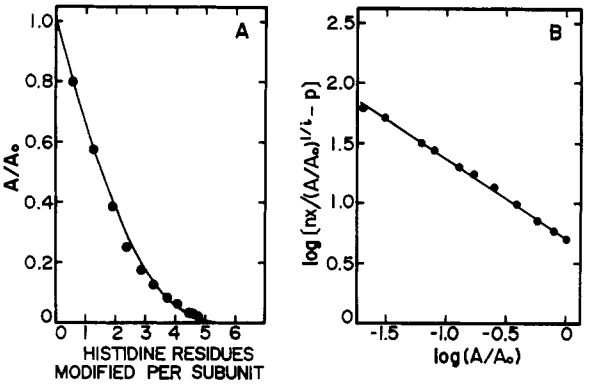

FIGURE 5: Relationship between the residual enzyme activity and the number of histidine residues modified. A cuvette containing the enzyme (48.4 nmol subunit) in 1.2 mL of 0.1 M potassium phosphate buffer, pH 6.9, and the one containing buffer alone were placed in the sample and reference compartments of a spectrophotometer maintained at 10 °C by circulating cold water. Three microliters of 0.3 M diethyl pyrocarbonate were added to both cuvettes, and the increase in absorbance at 242 nm was continuously measured. Aliquots were removed at intervals and assayed for enzyme activity. The number of histidine residues modified was calculated by using a difference absorbance of 3.2×10^3 M^{-1} cm^{-1}. (A) Plot of the residual enzyme activity against the number of residues modified. The *points* in the figure are experimental values, while the *line* is calculated by eq 3, with $n = 6$, $p = i = 1$, and $\alpha = 0.33$ (see below). (B) Tsou's analysis of the data. The data in (A) were plotted according to eq 4, with $n = 6$ and $p = i = 1$. See text for details.

single, symmetrical peak at the position expected for the native enzyme. Treatment of the eluate with hydroxylamine resulted in an activity peak which coincided with the protein peak (Figure 4).

S-Adenosylhomocysteinase, when excited at 280 nm, exhibits fluorescence with an emission maximum at 335 nm, characteristic of a tryptophan-containing protein. This intrinsic protein fluorescence was retained in the inactivated enzyme.

Thus, the results obtained above indicate that the inactivation of the enzyme by diethyl pyrocarbonate does not involve release of the bound NAD$^+$, or dissociation or aggregation of the enzyme, and also make an occurrence of gross conformational change unlikely.

Number of Histidine Residues Essential for Activity. Under the conditions of Figure 5, about five histidine residues per subunit were modified before complete inactivation. Prolonged incubation showed that a total of 6 out of 13 residues present per subunit (Fujioka & Takata, 1981) was modifiable by the reagent.

Figure 5A shows the relationship between the loss of enzyme

activity and the number of histidine residues modified. Extrapolation of the initial linear portion of the plot to zero enzyme activity shows that the modification of three residues is required for complete inactivation, but this method does not usually give the number of residues essential for activity (Tsou, 1962; Horiike & McCormick, 1979). Therefore, we have used the statistical method of Tsou (1962) to determine the number of essential residues.

If we assume that all of the modifiable residues including i essential residues are equally reactive toward the reagent and that the modification of any of the essential residues results in complete inactivation, the fraction of residues remaining, x, and that of activity remaining are related as

$$x = (A/A_0)^{1/i} \quad (2)$$

Therefore, a plot of x against the right-hand component of eq 2 should yield a straight line when i equals the number of essential residues. The plot of experimental data according to eq 2, however, did not give straight line with any value for i (i is a positive integer), showing that this assumption is not valid (not shown).

If, among n modifiable residues, p residues including i essential residues react with a given rate constant (k_1), and $n - p$ residues with a different rate constant ($k_2 = \alpha k_1$), the relationship of eq 3 will be obtained (Tsou, 1962) where m

$$m = n(1 - x) = n - p(A/A_0)^{1/i} - (n - p)(A/A_0)^{\alpha/i} \quad (3)$$

is the number of residues modified and x is the *total* fraction of residues remaining at a given stage of modification. Equation 3 can be rearranged to

$$\log [nx/(A/A_0)^{1/i} - p] = \log (n - p) + (\alpha - 1)/i \log (A/A_0) \quad (4)$$

As shown in Figure 5B, the plot of $\log [nx/(A/A_0)^{1/i} - p]$ against $\log (A/A_0)$ was a satisfactory straight line with $p = i = 1$ when $n = 6$. Any other combinations of values of p and i did not yield a straight line. The value of α obtained from the plot was 0.33. Thus, the result implies that only one histidine residue is essential for activity, and this residue reacts with diethyl pyrocarbonate about 3 times as fast as the nonessential residues.

Effect of Adenosine on Inactivation. It has been reported that diethyl pyrocarbonate reacts with adenosine with the opening of its imidazole ring (Leonard et al., 1971). Therefore, to test the effect of adenosine and *S*-adenosylhomocysteine on inactivation, we have first examined the reaction of these compounds with diethyl pyrocarbonate. When adenosine (50 μM) was incubated with 3 mM diethyl pyrocarbonate in 0.1 M potassium phosphate buffer at pH 6.9 and 5 °C, no significant change in its spectrum was observed during a 15-min incubation period. Furthermore, the compound after destruction of diethyl pyrocarbonate by heating at 100 °C for 10 min reacted normally with adenosine deaminase to form inosine. By contrast, *S*-adenosylhomocysteine appears to be very unstable under similar conditions. Whereas the spectrum was unaltered, the compound after the reaction with diethyl pyrocarbonate acted neither as substrate nor as inhibitor of *S*-adenosylhomocysteinase. The nature of the reaction of *S*-adenosylhomocysteine with diethyl pyrocarbonate and the identity of the reaction product(s) have not been explored.

The addition of 10 μM adenosine to the reaction mixture containing *S*-adenosylhomocysteinase and 2.2 mM diethyl pyrocarbonate retarded the rate of inactivation. The apparent first-order rate constant for inactivation at pH 6.9 and 0 °C was 0.09 min^{-1}, a value about half that of the unprotected control. No further protection was noted when the concentration of adenosine was increased above this level. As expected, the presence of *S*-adenosylhomocysteine (150 μM, 11 K_m) in the reaction mixture resulted in no significant change in the inactivation rate.

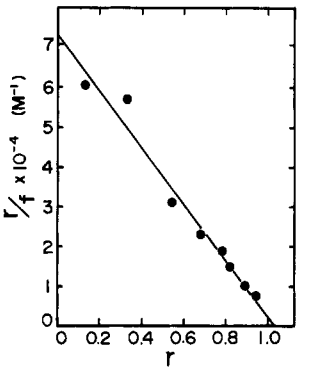

FIGURE 6: Scatchard plot of the binding of adenosine to the diethyl pyrocarbonate modified enzyme. The binding was measured by equilibrium dialysis method using 41.5 μM (subunit) modified enzyme and 1–120 μM [2,8-$^3H_2$]adenosine (4.4×10^6 dpm/μmol). The modified enzyme was prepared by incubating *S*-adenosylhomocysteinase with 3 mM diethyl pyrocarbonate for 30 min at pH 6.9 and 0 °C, followed by gel filtration through a Sephadex G-25 column (1.8×12 cm) to remove excess reagent. The residual enzyme activity was less than 1% of the initial value. r represents the number of moles of adenosine bound per mole of enzyme subunit and f the concentration of free adenosine. The line is drawn by a least-squares linear regression.

Properties of Modified Enzyme. The equilibrium dialysis study with [2,8-$^3H_2$]adenosine as ligand showed that the diethyl pyrocarbonate inactivated enzyme still retained the capacity to bind adenosine. A Scatchard plot of the binding data yielded a straight line and showed that each subunit was capable of binding 1 mol of adenosine (Figure 6). The apparent K_d value determined from the plot was 14.1 μM. The value with the native enzyme, determined previously under the same conditions, is 0.8 μM (Gomi & Fujioka, 1982).

Palmer & Abeles (1976, 1979) have shown that adenosine or *S*-adenosylhomocysteine is first oxidized to a 3′-keto derivative by the enzyme-bound NAD^+. The compound then undergoes proton abstraction at the 4′ position to form an α-ketocarbanion, which is converted to 3′-keto-4′,5′-dehydroadenosine after elimination of water or homocysteine. Since the diethyl pyrocarbonate inactivated enzyme still binds adenosine, the effect of modification on these partial reactions was examined. As with the native enzyme, the addition of adenosine to the inactivated enzyme caused an increase in absorbance at 327 nm due to the conversion of NAD^+ to NADH (Palmer & Abeles, 1976, 1976; Fujioka & Takata, 1981) (Figure 7A). However, the maximal change in absorbance [1.13×10^3 (M subunit)$^{-1}$ cm^{-1}] was about 32% of that found with the native enzyme. This value agrees with that obtained by the measurement of NADH in the native and modified enzymes after the addition of a saturating concentration of adenosine (200 μM) (data not shown). As shown in Figure 7B, *S*-adenosylhomocysteine also caused the appearance of this absorption in both the native and modified enzymes. The maximal changes in absorbance were 3.4×10^3 (M subunit)$^{-1}$ cm^{-1} for the former and 0.35×10^3 (M subunit)$^{-1}$ cm^{-1} for the latter.

The ability of the modified enzyme to catalyze the exchange of the 4′ proton with solvent was examined with [4′-^{3}H]-adenosine as substrate. The enzyme which had no detectable activity to catalyze the overall reaction catalyzed the release of radioactivity from the substrate at a rate 12% of the native enzyme. For elimination of the possibility that this slow exchange is due to the contaminating unmodified enzyme, the

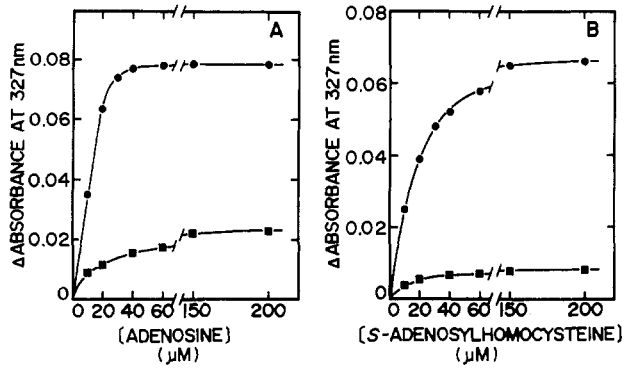

FIGURE 7: Increase in absorbance at 327 nm as a function of adenosine or *S*-adenosylhomocysteine concentration. Titration was carried out in 10 mM potassium phosphate buffer, pH 7.2, at 25 °C. (A) Titration with adenosine. (B) Titration with *S*-adenosylhomocysteine. Native enzyme (●) 21.3 μM subunit; modified enzyme (■) 23.8 μM subunit. The determinations of absorbance were made 30 min after each addition. The modified enzyme was prepared as described in Figure 6.

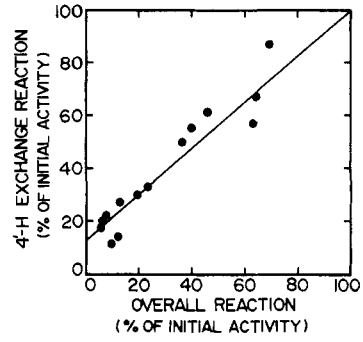

FIGURE 8: Comparison of the overall and 4'-proton exchange activities during inactivation. The enzyme (4 mg) in 3.5 mL of 0.1 M potassium phosphate buffer, pH 6.9, was incubated with 1.5 mM diethyl pyrocarbonate at 0 °C. At intervals, aliquots were removed, mixed with an equal volume of 40 mM imidazole at pH 7.5 (25 °C) to stop further inactivation, and assayed immediately for the overall activity and the 4'-proton exchange activity. The overall activity was determined both in the directions of *S*-adenosylhomocysteine hydrolysis and *S*-adenosylhomocysteine synthesis. The line is drawn by a least-squares linear regression.

percent activities remaining at various stages of modification were determined for the overall and exchange reactions (Figure 8). Clearly, the modification does not prevent the 4' proton exchange even though the rate is greatly diminished.

Discussion

S-Adenosylhomocysteinase from rat liver is inactivated rapidly by diethyl pyrocarbonate. The inactivation is not due to the modification of the bound NAD^+ or its release from the enzyme. The molecular size of the enzyme is also unaltered. Thus, the inactivation is due to the modification of amino acid residue(s) essential for catalytic action of the enzyme.

Diethyl pyrocarbonate reacts in a highly selective manner with histidine residues in a number of proteins, but the reaction with other nucleophilic residues is also reported [see, for review, Miles (1977)]. The reaction of *S*-adenosylhomocysteinase with [^{3}H]diethyl pyrocarbonate shows that, in addition to the modification of histidine residues, some label is incorporated into the enzyme in a hydroxylamine-stable manner. The hydroxylamine-stable modification is not related to the loss of enzyme activity. This together with the finding that hydroxylamine removes an amount of radiolabel equivalent to the number of histidine residues modified as determined spectrophotometrically indicates that the inactivation results from the modification of histidine. A pK value of 6.8 found for the inactivation (Figure 3) is also consistent with this conclusion.

The inactivation of *S*-adenosylhomocysteinase by diethyl pyrocarbonate proceeds in an all-or-none fashion. Quantitation of the number of histidine residues essential for activity by the method of Tsou (1962) indicates that only one among six modifiable residues is involved in the catalytic activity. The analysis also shows that the essential residue reacts with the reagent about 3 times more rapidly than nonessential residues. A rather small difference in reactivities of the essential and nonessential residues toward the reagent would make it impracticable to use the kinetic method of Ray & Koshland (1961) for analysis of the data.

The diethyl pyrocarbonate inactivation of *S*-adenosylhomocysteinase does not prevent the binding of adenosine and *S*-adenosylhomocysteine and the oxidation of these compounds at the 3' position as seen by the reduction of the bound NAD^+ (Figures 6 and 7). From the measurement of the NAD^+ and NADH contents, it has been previously shown that about 80% of NAD^+ is reduced when the native enzyme is exposed to excess adenosine (Gomi & Fujioka, 1982). Removal of excess adenosine with adenosine deaminase or gel filtration through Sephadex G-25 results in almost complete disappearance of the 327-nm peak. The same treatment of the mixture of the enzyme and *S*-adenosylhomocysteine also caused the disappearance of the peak. These findings indicate that, under the conditions, intermediates in the reaction pathway remain bound to the enzyme, and an equilibrium is established among enzyme species bearing various intermediates. Therefore, a decreased formation of NADH in the modified enzyme (Figure 7) is most simply explained by a shift in equilibrium positions as a result of modification.

With the modified enzyme, the increase in absorbance at 327 nm that occurs on the addition of adenosine or *S*-adenosylhomocysteine is slow, requiring about 30 min at 25 °C for maximal change. In contrast, an instantaneous reaction (complete within the time of mixing) occurs with the native enzyme. Since the oxidation of the 3'-hydroxyl group of substrate precedes the proton abstraction at the 4' position (Palmer & Abeles, 1979), the slow exchange of the 4' proton with solvent observed with the modified enzyme (Figure 8) does not necessarily mean that this process is slowed down.

In spite of the occurrence of partial reactions, the modified enzyme fails to produce homocysteine from *S*-adenosylhomocysteine. Therefore, it may be suggested that the inactivation is due to the abolition of the step which eliminates homocysteine from the putative 3'-ketocarbanion of *S*-adenosylhomocysteine and, if so, that the critical histidine is involved in the elimination/addition of homocysteine and water as an acid–base catalyst. It is also possible that the introduction of the carbethoxy group to the residue exerts an influence on the local conformation required for catalysis.

Acknowledgments

We thank Dr. T. Koizumi, Faculty of Pharmaceutical Sciences of this University, for helpful suggestions in the preparation of [^{3}H]diethyl pyrocarbonate.

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Steroid 21-Hydroxylase (Cytochrome P-450) from Porcine Adrenocortical Microsomes: Microsequence Analysis of Cysteine-Containing Peptides†

Pau-Miau Yuan, Shizuo Nakajin, Mitsuru Haniu, Masato Shinoda, Peter F. Hall, and John E. Shively*

ABSTRACT: The steroid 21-hydroxylase cytochrome P-450 from porcine adrenocortical microsomes was purified to homogeneity. The protein exhibited two NH_2-terminal sequences, one of which was identical with the first but lacking the NH_2-terminal methionine. The sequence was extremely hydrophobic but had little homology to the 17α-hydroxylase/$C_{17,20}$-lyase isolated from neonatal porcine testes or to rat or rabbit liver microsomal cytochromes P-450. The cysteine-containing fragments of the S-carboxymethylated protein were purified by high-performance liquid chromatography and sequenced. Three of the cysteine-containing peptides exhibited significant sequence homology with peptides from the major phenobarbital-induced rat liver cytochrome P-450 (P-450b) and two with peptides from cytochrome $P\text{-}450_{cam}$ (camphor methylene hydroxylase from *Pseudomonas putida*). The presence of conserved regions in the primary sequences of these proteins appears likely to provide clues to the nature of their heme-binding domains.

The conversion of cholesterol to active steroid hormones requires a number of enzymatic reactions of which several are catalyzed by cytochromes P-450 (Ryan & Engle, 1957; Estabrook et al., 1963). The steroidogenic cytochromes P-450 are characterized by well-defined substrate specificities and absolute specificity for the site on the substrate at which catalysis occurs (Nakajin & Hall, 1981). Because of the interest in enzymology of P-450 and the difficulty of working with drug metabolizing cytochromes P-450 in which substrate and position specificity are ill-defined, we have purified a number of the steroidogenic cytochromes P-450 in preparation for determination of their primary structures. To facilitate comparison between different steroidogenic cytochromes P-450, we have confined our studies to a single species, namely, the pig. Microsomal 21-hydroxylase was previously purified from beef adrenal (Kominami et al., 1980). We now report initial structural studies of cytochrome P-450, 21-hydroxylase from microsomes of pig adrenal glands.

Experimental Procedures

Materials. Acetonitrile (HPLC grade) was obtained from J. T. Baker Chemical Co. Trifluoroacetic acid was distilled first over chromium trioxide and then over alumina. Iodoacetic acid (Sigma) was recrystallized from petroleum ether prior to use, and iodo[1-^{14}C]acetic acid (50 μCi/0.7 mg) was obtained from New England Nuclear. 3-Sulfophenyl isothiocyanate (sodium salt) and *o*-phthaldialdehyde were from Pierce Chemical Co. Trypsin (TPCK treated; 232 units/mg) was obtained from Worthington Biochemical Co. *Staphylococcus aureus* protease (strain V8, specific activity 500–700 units/mg) was from Pierce Chemical Co. The sources of various chemicals for enzyme purification have been given elsewhere (Nakajin & Hall, 1981) except for the following: [4-^{14}C]-progesterone (lot no. 965-209; 55.7 mCi/mmol) and 17α-hydroxy[4-^{14}C]progesterone (lot no. 467-128; 50 mCi/mmol) were obtained from New England Nuclear Co. and hydroxylapatite was from Bio-Rad (Bio-Gel HTP).

Purification of 21-Hydroxylase. Pig adrenal glands were homogenized in 5 volumes of sucrose (0.25 M) containing 0.1 mM EDTA[1] and centrifuged at 9000*g* for 30 min. The su-

† From the Division of Immunology, City of Hope Research Institute, Duarte, California 91010 (P.-M.Y., M.H., and J.E.S.), the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545 (P.F.H.), and the Department of Biochemistry, Hoshi College of Pharmacy, Tokyo 142, Japan (S.N.). *Received July 27, 1982.* This investigation was supported by Grant CA 16434 from the National Cancer Institute and Grant AM-15621 from the National Institute of Arthritis and Metabolism.

0006-2960/83/0422-0143$01.50/0