Ligand-Dependent Changes in Intrinsic Fluorescence of *S*-Adenosylhomocysteine Hydrolase: Implications for the Mechanism of Inhibitor-Induced Inhibition†

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ABSTRACT: Different forms of *S*-adenosylhomocysteine (AdoHcy) hydrolase (NAD^+, apo, and NADH forms) were prepared, and the effects of ligand binding on the intrinsic tryptophan fluorescence were investigated. Binding of AdoHcy hydrolase (NAD^+ form) with its mechanism-based inhibitors [e.g., (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopentan-1′-yl)adenine] caused significant quenching (35%) of the intrinsic tryptophan fluorescence of the enzyme. A ligand-induced conformational change in the tertiary structure of the enzyme accounted for an initial 10% quenching of the fluorescence, with further fluorescence quenching occurring in a time-dependent manner. This time-dependent quenching of fluorescence was consistent with the time-dependent inactivation of the enzyme and the time-dependent reduction of the enzyme-bound NAD^+ to NADH. The time-dependent quenching of the intrinsic tryptophan fluorescence is largely the result of resonance energy transfer from tryptophan(s) in the enzyme to the enzyme-bound NADH. This interpretation is supported by the observations that the formation of enzyme-bound NADH quenched the intensity of the intrinsic tryptophan fluorescence and that the enzyme-bound NADH fluorescence was excited by light at wavelengths consistent with the absorption spectrum of tryptophan. Additional support for the involvement of NADH in the time-dependent tryptophan fluorescence quenching came from the observation that this quenching could only be observed when binding caused simultaneous reduction of the enzyme-bound NAD^+ to NADH. Binding of apo-AdoHcy hydrolase with mechanism-based inhibitors or binding of AdoHcy hydrolase (NAD^+ form) with 3′-keto-Ado or other 3′-modified Ado analogs that were incapable of reducing the enzyme-bound NAD^+ to NADH resulted in only 10% quenching of the intrinsic tryptophan fluorescence in a non-time-dependent manner. However, there was a significant contribution to the time-dependent quenching of tryptophan fluorescence that was associated with a conformational change caused by the conversion of the enzyme-bound NAD^+ to NADH, as indicated by the observed changes in the solvent accessibility of tryptophan to hydrophilic quenchers (e.g., KI) that varied between the NAD^+ and the apo forms of the enzyme after binding with the mechanism-based inhibitor. Data also indicated that there was at least one tryptophan near the active site of the enzyme and two tryptophans near the NAD^+ binding site. Inactivation of the AdoHcy hydrolase (NAD^+ form) by mechanism-based inhibitors has been shown previously to involve a cofactor depletion ($NAD^+ \rightarrow$ NADH) mechanism. Further evidence in support of this mechanism was obtained when it was observed that, upon inactivation of AdoHcy hydrolase (NAD^+ form) with mechanism-based inhibitors, the fluorescence lifetime of tryptophan(s) in AdoHcy hydrolase (NAD^+ form) approached the fluorescence lifetime of tryptophan(s) in the NADH form of the enzyme. When the NADH form of the enzyme was incubated with 3′-keto-Ado, the fluorescence of the enzyme-bound NADH was almost totally quenched and the intrinsic fluorescence of tryptophan was increased by 15%. A fluorescence method for rapid screening of potential AdoHcy hydrolase inhibitors was developed on the basis of these observations.

S-Adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and homocysteine (Hcy) (de la Haba & Cantoni, 1959). This enzyme has been an attractive target for the design of antiviral agents (De Clercq, 1987; Keller & Borchardt, 1988; Wolfe & Borchardt, 1991; Liu et al., 1992a) because of its important role in regulating biological methylation reactions by controlling intracellular levels of AdoHcy (Borchardt, 1980). Inhibition of this enzyme results in intracellular accumulation of AdoHcy and subsequent inhibition of all *S*-adenosylmethionine-dependent methylation reactions including viral mRNA methylations (Pugh et al., 1978; Borchardt & Pugh, 1979; Keller & Borchardt, 1986; Ransohoff et al., 1987; Hasobe et al., 1989).

In recent years, significant efforts have been made in designing potent and selective inhibitors of AdoHcy hydrolase (Borcherding et al., 1987, 1988; Madhavan et al., 1988; McCarthy et al., 1989; Wolfe & Borchardt, 1991; Jarvi et al., 1991; Robins et al., 1992; Liu et al., 1992a,b; Wolfe et al., 1992; Ramesh et al., 1992; Liu et al., 1993). The design of these potent and specific inhibitors of AdoHcy hydrolase has proceeded in spite of the lack of detailed information about the tertiary structure and the dynamics of this enzyme. Recently, progress has been made toward elucidation of the primary sequence of AdoHcy hydrolase (Ogawa et al., 1987; Coulter-Karis & Hershfield, 1989) and toward identification of key amino acid residues involved in substrate binding and/

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or catalysis (Aiyar & Hershfield, 1985; Gomi et al., 1986). Yeh et al. (1991) have taken advantage of this information to develop a molecular model of the active site and the NAD^+ binding site of the enzyme. This model proposes the existence of several tryptophan residues in the NAD^+ binding site of the enzyme. Therefore, we decided to undertake a series of experiments designed to determine whether inhibitor binding and/or enzyme inactivation resulted in changes in the intrinsic fluorescence of these tryptophan residues.

Intrinsic protein fluorescence due to endogenous tryptophan(s) is known to be sensitive to local microenvironments and has served as a useful probe for studying protein tertiary structure and its dynamics (Chen & Edelhoch, 1975). Measurement of the changes in intrinsic tryptophan fluorescence in a protein associated with the binding of ligands can be the most direct method to study ligand-induced conformational changes. The kinetic mechanism of AdoHcy hydrolase has been investigated recently using stopped-flow and intrinsic fluorescence quenching techniques (Porter & Boyd, 1991). In the present paper, the ligand-dependent intrinsic fluorescence changes in different forms of AdoHcy hydrolase (apo, NAD^+, and NADH) were studied to further demonstrate the mechanism of the enzyme inhibition, as well as to help refine the model for the tertiary structure around both the active site and the NAD^+ binding site.

MATERIALS AND METHODS

Materials. β-Ado, α-Ado, AdoHcy, and Ado deaminase were purchased from Sigma Chemical Co. (St. Louis, MO). The inhibitors, (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopentan-1′-yl)adenine (DHCaA), 3-deaza-DHCaA, 4′-α-methyl-DHCaA, 4′-β-methyl-DHCaA, 4′-β-vinyl-DHCaA, Ado 5′-carboxaldehyde, (1′*R*,2′*S*,3′*R*)-9-(2′,3′-dihydroxycyclopent-4′-enyl)adenine (DHCeA), and 3-deaza-DHCeA, were synthesized in our laboratory according to published procedures (Borcherding et al., 1987; Narayanan et al., 1988; Wolfe et al., 1992; Liu et al., 1993). (*Z*)-4′,5′-Didehydro-5′-deoxy-5′-fluoro-Ado (ZDDFA) was obtained from Dr. James R. McCarthy, Marion Merrell Dow Research Institute, Cincinnati, OH. 2′-Deoxy-2′-methylene-Ado (DOMA), (*Z*)-4′,5′-didehydro-5′-deoxy-5′-chloro-Ado (ZDDCA), 5′-fluoro-5′-*S*-(4-chlorophenyl)-5′-thio-Ado (5′*R*/5′*S* = 61/39) (FSCTAa), 5′-fluoro-5′-*S*-(4-chlorophenyl)-5′-thio-Ado (5′*R*/5′*S* = 38/62) (FSCTAb), and 3′-deoxy-3′-amino-Ado and 3′-deoxy-3′-cyclopropyl-Ado were obtained from Dr. Morris Robins, Department of Chemistry, Brigham Young University, Provo, UT.

Purification of Recombinant Rat Liver AdoHcy Hydrolase. The recombinant rat liver AdoHcy hydrolase (NAD^+ form) was purified from the cell-free extracts of *Escherichia coli* transformed with plasmid pPUCSAH and grown in the presence of isopropyl β-thiogalactopyranoside by the procedure of Gomi et al. (1989a), except that DEAE-Sepharose instead of DEAE-cellulose was used for ion-exchange chromatography (Yuan et al., unpublished experiments). The purified enzyme was homogeneous on SDS–PAGE and had a specific activity of 0.9–1.0 unit/mg. The purified enzyme contained 3.7 mol of tightly bound NAD^+, 0.3 mol of NADH per tetramer, and six tryptophan residues distributed throughout its 431-residue primary sequence (Ogawa et al., 1987).

Preparation of Apo-AdoHcy Hydrolase. Apo-AdoHcy hydrolase was obtained by treatment of AdoHcy hydrolase (NAD^+ form) with $(NH_4)_2SO_4$ to remove the enzyme-bound NAD^+. This method was a modification of that reported for the preparation of rat liver apo-AdoHcy hydrolase (Gomi et al., 1989b). In this experiment, 12 mg of recombinant rat liver AdoHcy hydrolase (NAD^+ form) was dissolved in 4 mL of 25 mM potassium phosphate (K_2HPO_4/KH_2PO_4) buffer, pH 7.2, containing 5 mM dithiothreitol (DTT) and 1 mM EDTA, and 8 mL of saturated $(NH_4)_2SO_4$ solution (pH 3.3), prepared by adjusting the pH of the potassium buffer described above with 3 N H_2SO_4, and was gradually added to the enzyme solution. After stirring for 30 min at 0° C, the precipitated apo-AdoHcy hydrolase was collected by centrifugation and dissolved in 4 mL of potassium phosphate buffer. The apo enzyme was precipitated again by addition of 8 mL of pH 3.3 $(NH_4)_2SO_4$ solution. The precipitate was dissolved in 4 mL of potassium phosphate buffer followed by washing with 8 mL of saturated neutral $(NH_4)_2SO_4$ containing 5 mM DTT and 1 mM EDTA. Finally, the precipitate was dissolved in 25 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA. Insoluble matter was removed by centrifugation, and the supernatant was apo-AdoHcy hydrolase. The apo enzyme prepared in the above procedure was inactive in the standard assay, but was converted to active enzyme by preincubation with 1 mM NAD^+ for 10 min at room temperature. The specific activity of the enzyme reconstituted with NAD^+ was typically 100–120% of that of the recombinant AdoHcy hydrolase (NAD^+ form). Presumably, recovery of greater than 100% activity was due to removal of any NADH form of the enzyme generated in the preparation and reconstitution of all the apo-AdoHcy hydrolase to active enzyme with NAD^+.

Preparation of AdoHcy Hydrolase (NADH Form). AdoHcy hydrolase (NADH form) was prepared by incubation of the above-prepared apo-AdoHcy hydrolase with 1 mM NADH for 30 min at room temperature. Excess NADH was removed by gel filtration on a Sephadex G-75 column (0.8 × 30 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA. This NADH form of AdoHcy hydrolase was inactive in the standard assay for AdoHcy hydrolase activity.

Assay of Enzyme Activity and Determination of Enzyme-Bound NAD^+ and NADH Content. The activity of AdoHcy hydrolase was assayed in the hydrolytic direction using [3,8-^{3}H]AdoHcy as substrate with the coupling of Ado deaminase as described previously (Matuszewska & Borchardt, 1987). The enzyme-bound NAD^+ and NADH content were determined by the fluorescence method as described previously (Hohman et al., 1985; Matuszewska & Borchardt, 1987).

Fluorescence Measurements. Fluorescence measurements were performed on a Perkin-Elmer-Hitachi 640-40 fluorescence spectrophotometer controlled by a 3600 data system station. Protein fluorescence was excited at 295 nm to minimize the contribution of protein tyrosine residues to the fluorescence (Eftink & Ghiron, 1976). Changes in protein fluorescence were monitored at the emission maximum of 344 nm. The slit widths on both the excitation and emission monochromators were set at 5 nm. Corrections for inner-filter effects due to the addition of ligand were not necessary since spectrophotometric examination of all the Ado analogs at the highest concentrations employed in this study revealed negligible absorption (<0.01 OD) at both the excitation (295 nm) and emission (344 nm) wavelengths. The sample compartment of the instrument was maintained at 25 °C with a circulated water bath. Fluorescence intensities reported here are relative values and are not corrected for wavelength variation in detector response.

For binding studies, the enzyme (concentration typically 30 μg/mL) was dissolved in 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA (buffer A). As the

fluorescence quenching of AdoHcy hydrolase by substrate or mechanism-based inhibitors was time-dependent, the fluorescence spectra were recorded at 3 min after addition of ligand unless stated otherwise, since at 3 min the maximum fluorescence quenching for most of the ligand used was approached. Ligands were introduced into the enzyme solution by adding 2–10 μL of stock solution of ligand to the cuvette and mixing immediately for 10 s using a small magnetic stirrer. Fluorescence changes due to addition of ligand volume were negligible as the dilution was less than 0.6%. The only variation to this methodology was in the studies involving 3′-keto-Ado, which were carried out in 20 mM HEPES buffer containing 1 mM EDTA, pH 7.0. This modification was necessary since 3′-keto-Ado is unstable in phosphate buffer at neutral pH (Porter & Boyd, 1992).

Titration of the enzyme with ligand was performed by adding the ligand to an enzyme solution; the fluorescence intensities were read at various time intervals. For each ligand concentration on the titration curve, a new enzyme solution was used. Data were corrected for loss of the fluorescence, presumbly due to photolysis of tryptophan or tyrosine (Applemann & Lienhard, 1985), by running a control sample in the absence of ligand for the same time period. Actually, the fluorescence loss due to photolysis was less than 1% in 1 min and 3–4% in 3 min.

Titration of the enzyme with small hydrophilic molecules such as potassium iodide and acrylamide was accomplished by making sequential additions from a stock solution of the quencher in buffer A to the enzyme, such that the total increase in volume was less than 3%. In each experiment, a control in which aliquots of buffer A, instead of quenching solution, were added to the cuvette was performed in order to determine the fluorescence changes that occurred as the result of dilution of the enzyme. Since the quenching effect of the ionic quencher I^- can be modified by the ionic strength and/or the effect of the counterion, a comparative experiment was performed with a nonquenching salt, KCl, and the necessary corrections were made. Quenching data were analyzed according to the modified Stern–Volmer plot (Leher, 1971; Samworth et al., 1988) by using the equation

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_{sv}[Q]} + \frac{1}{f_a} \quad (1)$$

where $\Delta F = F_0 - F_q$, F_0 is the intensity of fluorescence at a given wavelength in the absence of quenching agent, F_q is the intensity of fluorescence at the same wavelength in the presence of a known concentration [Q] of quenching agent, K_{sv} is the Stern–Volmer quenching constant, and f_a is the fraction of the initial fluorescence which is accessible to the quencher. A plot of $F_0/\Delta F$ *vs* $1/[Q]$ yields f_a^{-1} as the *y*-intercept and $(f_a K_{sv})^{-1}$ as the slope. For pH titration experiments, the sample pH was titrated with 0.5 N HCl and measured directly in the cuvette with a pH electrode.

Fluorescence Lifetime Measurement. Fluorescence lifetimes were determined using a multifrequency phase fluorometer (Model-ISS K_2). The sample temperature was 10 °C. Excitation was provided at 298 nm with a 300-W xenon lamp using a 8-nm slitwidth. Higher harmonics were removed using a Schott UG 11 broadband filter. The excitation light was intensity modulated using a Pockels cells, and phase and modulation data were collected at 12 frequencies evenly spaced between 2 and 200 MHz on a logarithmic scale. Fluorescence emission was detected using a Schott WG320 long-pass filter in conjunction with a Corning 7-51 broadband filter, and the fluorescence intensity decay was analyzed as a sum of exponentials. The impulse response $I(t)$ is given by

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

where α_i is the preexponential factor and τ_i is the decay time. The relationship between the measured phase angle and modulation of the sample has previously been defined (Weber, 1977; Lakowicz, 1986), and the parameters describing the fluorescence intensity decay were obtained from a nonlinear least-squares fit to the impulse response function (Bevington, 1969) by minimizing the χ_R^2:

$$\chi_R^2 = \frac{1}{\nu}\sum_\omega \left(\frac{\phi_\omega - \phi_{c\omega}}{\delta\phi}\right)^2 + \frac{1}{\nu}\sum_\omega \left(\frac{m_\omega - m_{c\omega}}{\delta m}\right)^2$$

where ν is the number of degrees of freedom and $\delta\phi$ and δm are the experimental uncertainties in the measured phase and modulation values, and are 0.4° and 0.01°, respectively. ω and c refer to the modulation frequency and calculated phase (ϕ) and modulation (m) values, respectively. In all cases a two-component fit ($\chi_R^2 = 3.6$) was justified to describe the data, as judged by the 25-fold reduction in the χ_R^2 in comparison to a single-exponential model. In contrast, the improvement in the χ_R^2 for a three-component model ($\chi_R^2 = 2.4$) was not significantly improved.

The fractional intensity (f_i) of each component of the emission is given by

$$f_i = \alpha_i\tau_i / \sum_i \alpha_i\tau_i$$

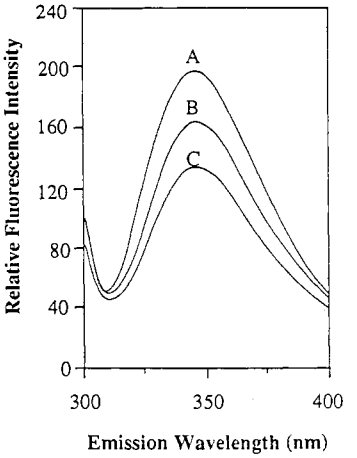

FIGURE 1: Fluorescence emission spectra of recombinant rat liver AdoHcy hydrolase (NAD^+ form). The enzyme in buffer A at 25 °C in the absence or presence of ligand (AdoHcy or DHCaA, 100 μM) was excited at 295 nm. The spectra were recorded at 3 min after addition of the ligand. Curve A is the free AdoHcy hydrolase (NAD^+ form), curve B is the NAD^+ form of enzyme + AdoHcy, and curve C is the NAD^+ form of enzyme + DHCaA.

RESULTS

Intrinsic Tryptophan Fluorescence of AdoHcy Hydrolase (NAD^+ Form). When excited at 295 nm, the intrinsic tryptophan fluorescence of AdoHcy hydrolase (NAD^+ form) showed an emission maximum at 344 nm (Figure 1). Since typical emission maxima of tryptophan are around 350 nm in water and 310–324 nm in nonpolar protein regions (Konev, 1967), this would indicate that most of the tryptophan emission in the recombinant rat liver AdoHcy hydrolase resulted from residues in relatively polar environments, suggesting that they are either near the protein surface or adjacent to internal salt binding with the protein. When an

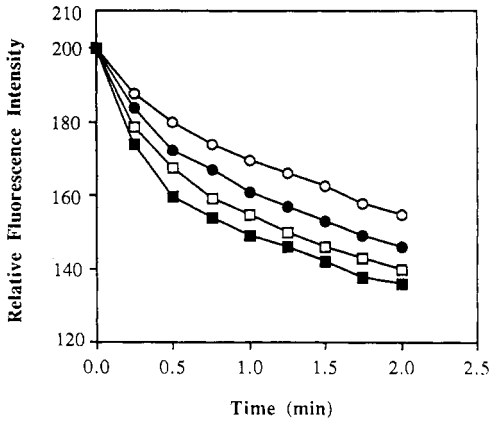

FIGURE 2: Time- and concentration-dependent intrinsic tryptophan fluorescence quenching of recombinant rat liver AdoHcy hydrolase (NAD^+ form) by DHCaA. Different concentrations of the inhibitor (DHCaA) (○, 1.25 μM; ●, 2.5 μM; □, 5 μM; and ■, 7.5 μM) were introduced into a solution of the enzyme (30 μg/mL) in buffer A at 25 °C, and the fluorescence changes were measured at various time intervals with an excitation wavelength of 295 nm and an emission wavelength of 344 nm.

excess of a substrate (AdoHcy) or a mechanism-based inhibitor (DHCaA) was added to the enzyme solution, the fluorescence was quenched significantly (Figure 1). The emission spectrum peak height was diminished by approximately 20% by addition of 100 μM of AdoHcy and 35% by addition of 100 μM of DHCaA (K_i = 156 nM, K_{inact} = 0.09 min^{-1} at 25 °C). It should be noted that neither the substrate nor the inhibitor caused a noticeable shift in the maximum emission wavelength. This indicates that the quenching induced by substrate or inhibitor was due to a reduction in the quantum yield (Pawagi & Deber, 1990) and was not the result of a shift of tryptophan residues from polar to nonpolar environments or vice versa.

Quenching of the intrinsic tryptophan fluorescence in AdoHcy hydrolase by DHCaA was shown to be concentration- and time-dependent (Figure 2). The extent of fluorescence quenching (% of $\Delta F/\Delta F_{max}$) correlated with the enzyme inactivation and the cofactor depletion ($NAD^+ \rightarrow NADH$) (Figure 3), indicating that the changes in fluorescence provide a direct and sensitive means to access the enzyme conformational properties and/or reduction of the enzyme-bound NAD^+.

The ligand-induced fluorescence quenching was specific for known substrates and inhibitors. For example, 100 μM β-Ado, a substrate of the enzyme in the synthetic direction but an inhibitor in the hydrolytic direction, quenched the fluorescence by 25%. In contrast, α-Ado, which is neither a substrate nor an inhibitor of the enzyme, had little effect on the fluorescence (data not shown). The specificity of Ado for the β configuration indicated a strict steric requirement for the C-1′ configuration in enzyme–ligand binding. It was also consistent with the fact that the fluorescence quenched by β-Ado could be partially recovered by addition of Hcy (data not shown). This is because β-Ado in the presence of Hcy will generate AdoHcy in the enzyme active site, thus allowing the enzyme to return to the catalytically active form (NAD^+ form). The fluorescence quenched by DHCaA could not be recovered either by the addition of Hcy or by dialysis (data not shown), suggesting that the inhibitor was trapped by the enzyme and, in turn, kept the enzyme in the NADH form.

Intrinsic Tryptophan Fluorescence of Apo and NADH Forms of AdoHcy Hydrolase. Figure 4 shows the emission spectra of tryptophan fluorescence of different forms of AdoHcy hydrolase excited at 295 nm. All three forms of

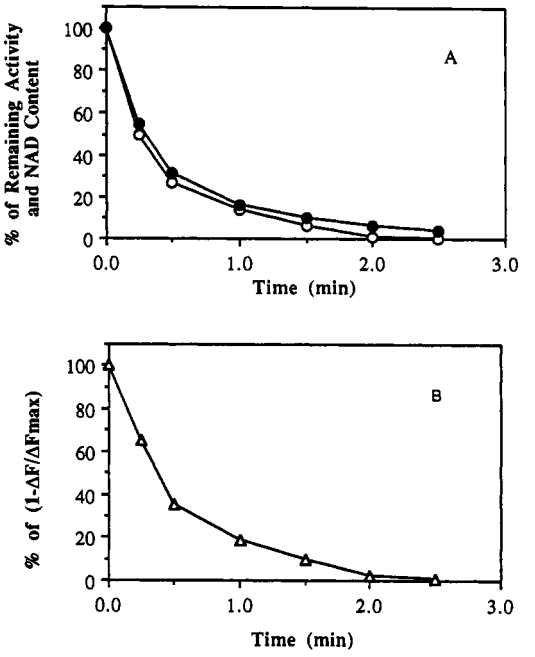

FIGURE 3: Correlations between enzyme inactivation, NAD^+ depletion, and intrinsic tryptophan fluorescence quenching of AdoHcy hydrolase (NAD^+ form) treated with DHCaA. AdoHcy hydrolase (NAD^+ form) was incubated with DHCaA (20 μM) at 25 °C in buffer A for different times. The remaining activity (panel A, ○) and NAD^+ content (panel A, ●) were assayed as described in Materials and Methods, and the fluorescence quenching (panel B, △) was measured at an excitation wavelength of 295 nm and an emission wavelength of 344 nm. The ΔF_{max} was obtained from a double-reciprocal plot of $1/\Delta F$ *vs* $1/[ligand]$ by extrapolating to infinite concentration of ligand (Ward, 1985).

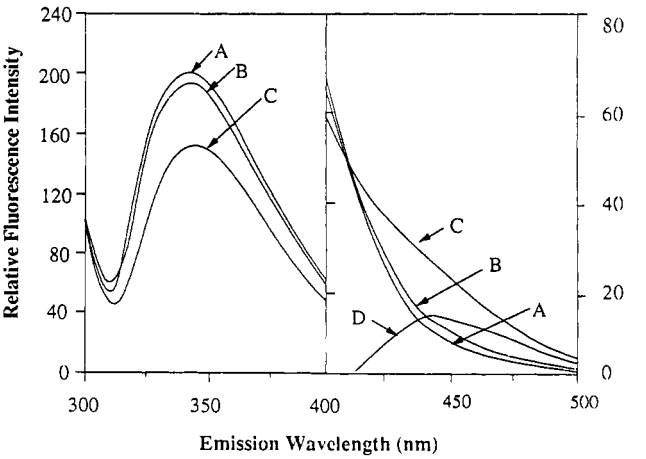

FIGURE 4: Fluorescence emission spectra of the NAD^+, NADH, and apo forms of recombinant rat liver AdoHcy hydrolase excited at 295 nm. Protein concentrations were 30 μg/mL in buffer A. Curve A is the apo-AdoHcy hydrolase, curve B is the NAD^+ form of the enzyme, curve C is the NADH form of the enzyme, and curve D is the difference spectrum, curve C – curve B. Curve D corresponds to the contribution of the enzyme–bound NADH to the total emission excited at this wavelength.

AdoHcy hydrolase (apo, NAD^+, and NADH) had strong tryptophan emission bands with maxima at 344 nm, indicating that the environment associated with the tryptophans is unchanged. The relative fluorescence intensity of the Apo and NADH forms of the enzyme was 108% and 78% relative to the catalytic active NAD^+ form. Since the three forms of the AdoHcy hydrolase showed no difference in either the secondary structure based on CD spectra analysis (data not shown) or in the emission mxaimum of tryptophan fluorescence, the reason for the differences in tryptophan fluorescence

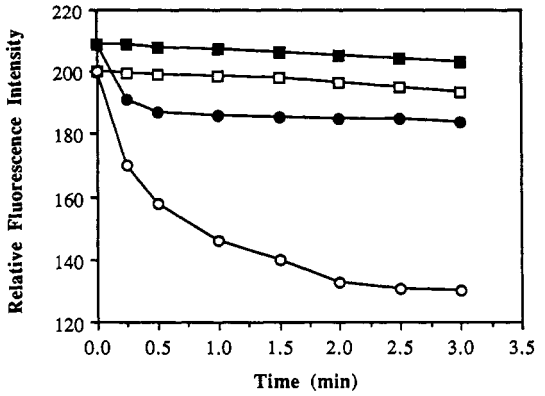

FIGURE 5: Quenching of the intrinsic tryptophan fluorescence of recombinant rat liver AdoHcy hydrolase (apo and NAD^+ forms) by DHCaA. Fluorescence was measured at 25 °C in buffer A at different time intervals, with an excitation wavelength of 295 nm and an emission wavelength of 344 nm. Protein concentrations were 30 μg/mL: (■) apo-AdoHcy hydrolase; (□) NAD^+ form of AdoHcy hydrolase; (●) apo-AdoHcy hydrolase + 20 μM DHCaA; (○) NAD^+ form of AdoHcy hydrolase + 20 μM DHCaA.

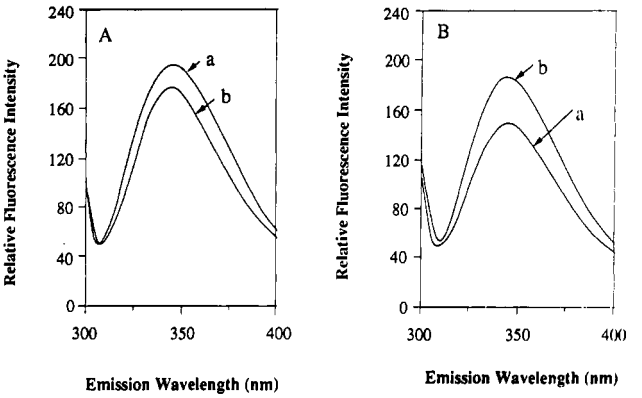

FIGURE 6: Fluorescence emission spectra of recombinant rat liver AdoHcy hydrolase in the absence or presence of 3′-keto-Ado. (Panel A) NAD^+ form of AdoHcy hydrolase in the absence (curve a) and presence (curve b) of 3′-keto-Ado (20 μM). (Panel B) NADH form of AdoHcy hydrolase in the absence (curve a) and presence (curve b) of 3′-keto-Ado (20 μM). Fluorescence was measured at 25 °C in 20 mM HEPES buffer containing 1 mM EDTA, pH 7.0, with excitation wavelength at 295 nm.

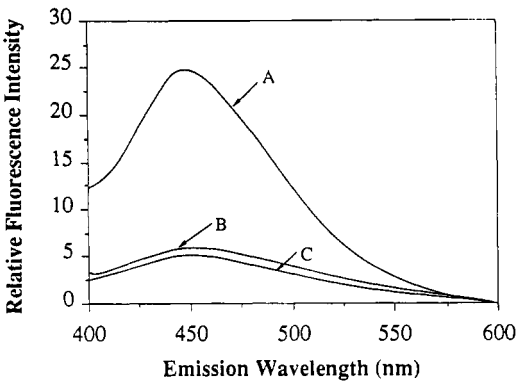

FIGURE 7: Fluorescence quenching of recombinant rat liver AdoHcy hydrolase (NADH form) by 3′-keto-Ado. Fluorescence was measured at 25 °C in 20 mM HEPES buffer containing 1 mM EDTA, pH 7.0, after a 10-min preincubation of the enzyme in the absence (curve A) or presence (curve B) of 20 μM 3′-keto-Ado. Curve C is the NAD^+ form of the enzyme only. The excitation wavelength was 340 nm.

intensity could be due to the difference in content of the enzyme-bound NADH among the three forms of the enzyme. Since the purified recombinant rat liver AdoHcy hydrolase (NAD^+ form) always contained a small amount of NADH (6–8% of total enzyme-bound nucleosides; a direct proof for this is shown on Figure 7, curve C), the fluorescence intensity was always slightly lower than the apo enzyme. It should be noted that, at wavelengths longer than 410 nm, the emission of the NADH form of the enzyme was enhanced (Figure 4) at the excitation wavelength of 295 nm. Difference spectra, i.e., the NADH form of the enzyme (curve C) minus the NAD^+ form of the enzyme (curve B), established the enhancement in the 440-nm region as arising from emission by bound NADH (curve D). The effect of the enzyme-bound NADH on the intrinsic tryptophan fluorescence was also confirmed by the different fluorescence lifetimes (τ) of tryptophan(s) among the three forms of AdoHcy hydrolase. The average fluorescence lifetimes (τ) of tryptophan(s) in the apo and NAD^+ forms of the enzyme were found to be 4.69 and 4.21 ns, respectively, which were longer than the average lifetime of tryptophan fluorescence in the NADH form of the enzyme (3.45 ns). This 18% decrease in the average fluorescence lifetime of tryptophan(s) associated with the NADH form of the enzyme relative to the NAD^+ form of the enzyme was comparable to the 22% decrease in the fluorescence intensiy. When the NAD^+ form of AdoHcy hydrolase was inactivated by the mechanism-based inhibitor (DHCaA), the average lifetime of tryptophan(s) fluorescence dropped to 3.66 ns, which is close to the lifetime of tryptophan fluorescence in the NADH form of the enzyme, indicating the conversion of the enzyme-bound NAD^+ to NADH after the binding of the enzyme and inhibitor.

The binding of inhibitors to apo-AdoHcy hydrolase caused non-time-dependent tryptophan fluorescence quenching of 10–11% (Figure 5). This is in contrast to the NAD^+ form of the enzyme which, when treated with a mechanism-based inhibitor (DHCaA), showed time-dependent quenching of tryptophan fluorescence for at least 3 min and a reduction of fluorescence intensity of approximately 35%.

Binding of 3′-Keto-Ado to AdoHcy Hydrolase (NAD^+ and NADH Forms). 3′-Keto-Ado is a key intermediate in the mechanism of the catalytic reaction of AdoHcy hydrolase as proposed by Palmer and Abeles (1979). It is formed by the enzymatic oxidation at the 3′-hydroxyl group of Ado by reduction of enzyme-bound NAD^+ to NADH. When 3′-keto-Ado was added to the NAD^+ form of AdoHcy hydrolase, tryptophan fluorescence was quenched by 9% (Figure 6A) and the quenching was not time-dependent. Similar results were observed when the NAD^+ form of the enzyme was treated with inhibitors whose 3′-hydroxyl group had been modified (e.g., 3′-deoxy-3′-amino-Ado and 3′-deoxy-3′-cyclopropyl-Ado) so that they could not be oxidized by the enzyme (data not shown). In contrast, when 3′-keto-Ado was added to the NADH form of AdoHcy hydrolase, the tryptophan fluorescence intensity was increased by about 15% (Figure 6B). This result would suggest that the 3′-keto-Ado was in part reduced by the NADH form of the enzyme, resulting in the generation of the NAD^+ form of the enzyme. As shown previously, the NAD^+ form of the enzyme has a higher tryptophan fluorescence intensity than does the NADH form of the enzyme. The oxidation of NADH to NAD^+ by 3′-keto-Ado could also be confirmed by taking emission fluorescence spectra of enzyme-bound NADH at an excitation wavelength of 340 nm (where NADH is directly excited), as shown in Figure 7. The enzyme-bound NADH showed a maximum emission wavelength around 440 nm. The NADH fluorescence of AdoHcy hydrolase (NADH form) was quenched to the level of the NAD^+ form of the enzyme upon binding with 3′-keto-Ado

Table I: Quenching of Intrinsic Tryptophan Fluorescence in Recombinant Rat Liver AdoHcy Hydrolase (Apo and NAD^+ Forms) by Hydrophilic Quenchers in the Absence and Presence of an Inhibitor (DHCaA)[a]

	% of quenching in protein fluorescence		ligand-induced % change in quenching ability	
sample	+KI	+acrylamide	+KI	+acrylamide
apo form	37.8	43.9		
NAD^+ form	37.4	43.2		
apo form + DHCaA	46.2	54.3	+8.4	+10.4
NAD^+ form + DHCaA	49.5	58.7	+12.1	+15.5

[a] Protein concentration: 30 μg/mL; ligand concentration: 100 μM; hydrophilic quencher concentration: 200 mM. Fluorescence was measured at 25 °C in buffer A. Excitation wavelength was 295 nm and emission wavelength 344 nm. Data were the average of duplicate measurements.

(Figure 7). This result was in agreement with that reported for the fluorescence change in bovine liver AdoHcy hydrolase due to the conversion of NADH to NAD^+ (Porter & Boyd, 1992).

Quenching of the Intrinsic Tryptophan Fluorescence of AdoHcy Hydrolase (NAD^+ Form) by Collisional Quenchers. To investigate the environment of the tryptophan residues in AdoHcy hydrolase, two hydrophilic collisional quenching agents (KI, an ionic quencher, and acrylamide, a neutral quencher) were used to test the accessibility of tryptophan in the enzyme. The tryptophan fluorescence intensities in the apo and NAD^+ forms of AdoHcy hydrolase were almost identically quenched by 37–38% by KI and 43–44% by acrylamide at 200 mM quencher (Table I). This suggests that the apo and the NAD^+ forms of the enzyme have similar conformations. These data also indicate that about 60% of the total protein tryptophan fluorescence was not available for quenching by KI and acrylamide at this concentration, which suggests that these inaccessible tryptophan residues are buried in the protein matrix. The binding of the mechanism-based inhibitor (DHCaA) to apo-AdoHcy hydrolase significantly increased the fluorescence quenching, 8.4% by KI and 10.4% by acrylamide (Table I), indicating that the conformational change induced by ligand binding may cause some tryptophan residues to become more exposed and thus more accessible to the hydrophilic quenchers. When the inhibitor (DHCaA) was added to the NAD^+ form of the enzyme, the quenching abilities were increased by 12.1% by KI and 15.5% by acrylamide (Table I). These differences in the increase in quenching ability between the apo and NAD^+ forms of the enzyme after binding the inhibitor indicate that a secondary conformational change may have occurred when the NAD^+ form of the enzyme was converted to the NADH form by the oxidation of the inhibitor. This conformational change may bring some tryptophans from the interior to the surface of the enzyme, thus increasing their accessibility to the hydrophilic quenchers.

The modified Stern–Volmer plots for KI quenching of the apo and NAD^+ forms of AdoHcy hydrolase in the absence and presence of the inhibitor (DHCaA) are shown in Figure 8. In the absence of the inhibitor (lines a), the K_{sv} and f_a values in both the NAD^+ form (panel A in Figure 8) and the apo form of the enzyme (panel B in Figure 8) were similar, with K_{sv} values of 8.6 M^{-1} and 8.8 M^{-1} and f_a values of 0.55 and 0.56 for the NAD^+ form and the apo form of the enzyme, respectively. In contrast, in the presence of the inhibitor (lines b), the K_{sv} and f_a values (12.4 M^{-1} and 0.76) for the NAD^+ form of the enzyme were larger than the values (11.2 M^{-1} and 0.71) for the apo enzyme. The values of f_a from these analyses indicate that, at infinite quencher concentration ($1/[Q] = 0$), approximately three tryptophan residues per subunit in both the apo and NAD^+ forms of the enzyme are accessible to the quencher (KI), assuming that the quantum yields of all six tryptophan residues are identical. After binding with the mechanism-based inhibitor (DHCaA), the K_{sv} and f_a values for the NAD^+ form of the enzyme were larger than those for the apo form, indicating that a conformational change resulted from the conversion of the NAD^+ form of the enzyme to the NADH form. Using the equation $k_q = K_{sv}/\tau$, the bimolecular quenching constants (k_q) in the absence of the inhibitor were calculated to be 2.0×10^9 M^{-1} s^{-1} for the NAD^+ form of the enzyme and 1.9×10^9 M^{-1} s^{-1} for the apo form of the enzyme. In the presence of the inhibitor, the NAD^+ form of the enzyme is converted to the NADH form, and the k_q value increased to 3.5×10^9 M^{-1} s^{-1}, indicating a substantial conformational change.

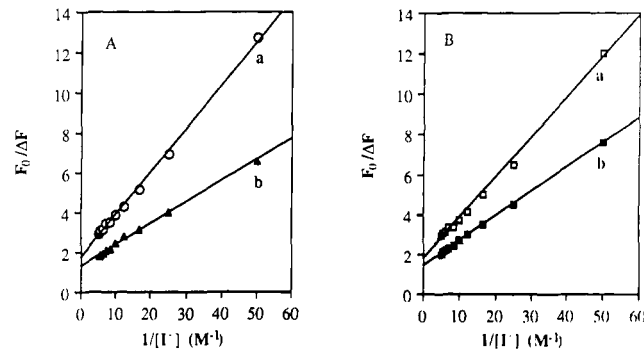

FIGURE 8: Modified Stern–Volmer plots for quenching tryptophan fluorescence in recombinant rat liver AdoHcy hydrolase (NAD^+ and apo forms) by KI. Protein concentrations were 30 μg/mL. Fluorescence was measured at 25 °C in buffer A. The excitation wavelength was 295 nm, and the emission wavelength was 344 nm. The enzymes were titrated with KI under the conditions described in Materials and Methods, and the data were plotted according to a modified Stern–Volmer equation (1) that permits calculation of a binding constant and the fraction of fluorophores available to the quenching agent. Appropriate corrections were made for dilutions during the titration. (Panel A) Line a is the NAD^+ form of AdoHcy hydrolase, and line b is the NAD^+ form of the enzyme + DHCaA (100 μM). (Panel B) Line a is the free apo-AdoHcy hydrolase, and line b is the apo enzyme + DHCaA (100 μM).

Internal Quenching of the Intrinsic Tryptophan Fluorescence of AdoHcy Hydrolase (NAD^+ Form) at Varying pH. Intrinsic tryptophan fluorescence in proteins is known to be sensitive to protonation or deprotonation of neighboring residues such as the imidazolium group of histidine (pK_a 5.6–7.0), the carboxyl groups of aspartic acid (pK_a 3.0–4.7) and glutamic acid (pK_a around 4.4), or the sulfhydryl group of cysteine (pK_a 8.5–11) (Stryer, 1975; White et al., 1978). Figure 9 shows the pH titration curves of the intrinsic tryptophan fluorescence of AdoHcy hydrolase (NAD^+ form) in the absence and presence of substrates or an inhibitor. The fluorescence of polar tryptophan residues in AdoHcy hydrolase (NAD^+ form) was affected by changes in pH between 3.5 and 7.2 with an apparent inflection point around pH 5.2. Approximately 15% of the fluorescence was quenched over the pH region from 7.2 to 5.2. This suggests that one tryptophan could be adjacent to a histidine residue and be quenched by its protonation. In contrast, in the presence of an excess of a substrate or an inhibitor, the fluorescence was only slightly affected by changes over the pH region 7.0–5.0 (Figure 9). This would indicate that the tryptophan was moved away from the histidine or, alternatively, that the tryptophan fluorescence had already been quenched by NADH generated

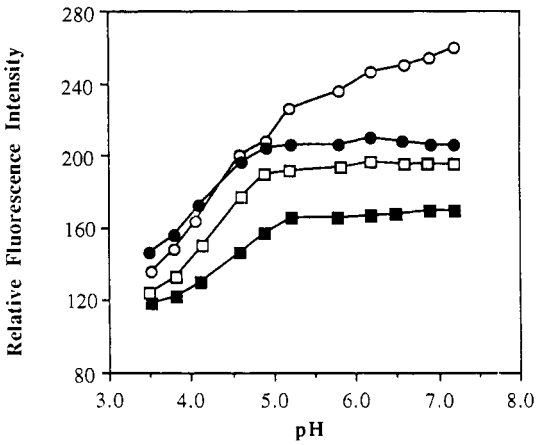

FIGURE 9: pH dependence of the intrinsic tryptophan fluorescence of recombinant rat liver AdoHcy hydrolase (NAD^+ form). The fluorescence was measured at an excitation wavelength of 295 nm and emission wavelength of 344 nm, at 25 °C. The pH titration was accomplished after 10 min of preincubation of AdoHcy hydrolase (40 μg/mL) at 25 °C in the absence (○) and presence of one of the following ligands: 100 μM AdoHcy (●), 100 μM Ado (□), and 100 μM DHCaA (■).

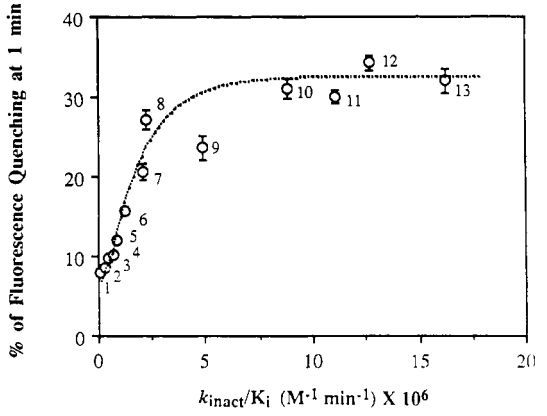

FIGURE 10: A plot of percent (%) of fluorescence quenching *vs* second-order rate constant (k_{inact}/K_i) of AdoHcy hydrolase inhibitors. Fluorescence quenching was measured at 25 °C in buffer A at the first minute after addition of inhibitors to a final concentration of 20 μM. The excitation wavelength was 295 nm, and the emission wavelength was 344 nm. Protein concentration was 30 μg/mL. Inhibition constants (k_{inact} and K_i) were determined at 37 °C in the synthetic direction using HPLC (Wolfe et al., 1992). The inhibitors are as follows: **1**, 4′-α-methyl-DHCaA; **2**, DOMA; **3**, 3-deaza-DHCeA; **4**, DHCeA; **5**, 4′-β-methyl-DHCaA; **6**, 4′-β-vinyl-DHCaA; **7**, 3-deaza-DHCaA; **8**, DHCaA; **9**, ZDDFA; **10**, ZDDCA; **11**, FSCTAa; **12**, FSCTAb; **13**, Ado 5′-carboxaldehyde.

by the ligand binding. The fluorescence quenching in the lower pH region (3.5–5.0) could be due to the protonation of a carboxyl group nearby the tryptophan residues.

A Fluorescence Method for Rapid Screening and Evaluation of AdoHcy Hydrolase Inhibitors. The fact that the intrinsic tryptophan fluorescence quenching process reflects the mechanism of the enzyme inactivation has allowed us to develop a fluorescence method for rapid screening and evaluation of AdoHcy hydrolase inhibitors. Since the enzyme inactivation by mechanism-based inhibitors is time-dependent, a time-dependent fluorescence quenching is observed. The more potent the inhibitor, the more fluorescence quenching is expected within a given time period. The fluorescence quenching data taken after 1 min of incubation appear to be ideal for this screening purpose, since most of the known inhibitors inactivate the enzyme by 30–70% in this time period at 25 °C. Figure 10 shows a plot of $\%(\Delta F_1)_{min}$ *vs* k_{inact}/K_i of various known inhibitors tested earlier in our laboratory. It is clear that the more the tryptophan fluorescence is quenched, the greater is the second-order rate constant of enzyme inhibition. If a new compound shows fluorescence quenching (ΔF) of 20% in 1 min, an approximate value of (2–3) × 10^6 M^{-1} min^{-1} for its second-order rate constant (k_{inact}/K_i) is easily obtained from Figure 10. Moreover, this is also a rapid and convenient way to tell whether or not a compound is a mechanism-based inhibitor of AdoHcy hydrolase.

DISCUSSION

The fluorescence quantum yield observed for the recombinant rat liver AdoHcy hydrolase is expected to reflect the summation of the yields of all the six tryptophan residues in the protein and will be affected by perturbation in local microenvironments. In this study, we have shown that the quenching of the intrinsic tryptophan fluorescence of AdoHcy hydrolase (NAD^+ form) upon binding with mechanism-based inhibitors may result from two different contributing factors: (1) a conformational change in the ligand-bound state which alters interactions of specific tryptophan residues with neighboring groups; and (2) the energy transfer from tryptophan to the enzyme-bound NADH. These two contributions to the fluorescence quenching have been distinguished from each other by using different forms of AdoHcy hydrolase and different ligands.

Quenching as a Consequence of Conformational Change. Binding of mechanism-based inhibitors to the apo form of the enzyme caused an approximate 10% decrease in the intrinsic tryptophan fluorescence in a non-time-dependent manner. This suggests that in the absence of the enzyme cofactor NAD^+ the enzyme can still bind the inhibitor and results in a conformational change corresponding to 10% fluorescence quenching. This may also indicate that there is one exposed tryptophan which is near the active site. This ligand-induced local protein conformational change is also supported by the observation that the ligand-bound state increases the accessibility to hydrophilic quenchers and decreases the sensitivity to pH-induced fluorescence quenching. Further evidence in support of this conformational change is the observation that 3′-keto-Ado or inhibitors which have their 3′-hydroxyl group modified (e.g., 3′-deoxy-3′-amino-Ado and 3′-deoxy-3′-cyclopropyl-Ado) also quench only about 10% of the tryptophan fluorescence in the NAD^+ form of the enzyme. Hence, this conformational change, which causes 10% fluorescence quenching, may be essential to the enzyme–ligand complex formation even without occurrence of any catalytic turnover.

Quenching as a Consequence of Energy Transfer. Intrinsic fluorescence in a protein can be quenched by its prosthetic group(s) due to resonance energy transfer if this group is within a limited distance and the absorbance spectrum of the prosthetic group overlaps the emisssion spectrum of tryptophan (Stryer, 1960). NADH has a maximum absorbance wavelength around 340 nm which overlaps the maximum emission wavelength of tryptophan (344 nm). The occurrence of energy transfer from tryptophan to protein-bound NADH was well demonstrated earlier in lactic dehydrogenase (LDH) (Velick, 1958). It has been shown that if fluorescence energy transfer occurs, there are two basic features observed: the decrease in the amount of the fluorescence of the energy donor, and the excitation of the acceptor fluorescence by light absorbed by the donor. These two features have been clearly shown in the present work for recombinant rat liver AdoHcy hydrolase. The formation of enzyme-bound NADH does decrease the amount of tryptophan fluorescence and increase the intensity of NADH fluorescence (λ_{max} = 440 nm) when tryptophan is selectively excited (Figure 4). This increase in the

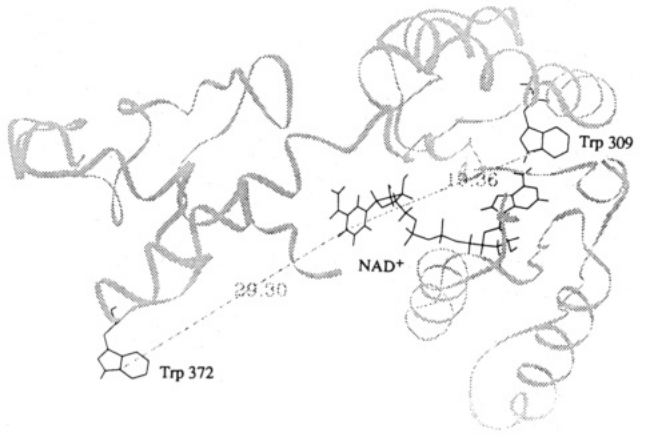

FIGURE 11: A computer model of the NAD^+ binding site of recombinant rat liver AdoHcy hydrolase.

fluorescence intensity of NADH upon selective excitation of tryptophan indicates that the change in the fluorescence intensity of tryptophan is at least in part a result of fluorescence resonance energy transfer from tryptophan to NADH. The weak emission of NADH fluorescence (curve D in Figure 4) may be due to the low quantum yield (2%) of NADH fluorescence (Weber, 1957). One may approximate the expected energy transfer efficiency between tryptophan and NADH using a computer graphics model of this recombinant rat liver AdoHcy hydrolase generated by our laboratory (Yeh et al., 1991). There are two tryptophan residues (Trp 309 and Trp 372) which are located close to the NAD^+ binding site (Figure 11). The distances (R measured from the three-dimensional graphics model) from Trp 309 and Trp 372 to the enzyme-bound NAD^+ are estimated to be 19.36 and 29.30 Å, respectively. Karreman et al. (1958) calculated from Förster's theory that energy transfer should occur between tryptophan and NADH with an R_0 value of 25 Å (R_0 is the distance between an energy donor and an acceptor molecule over which excitation energy may be transferred by resonance with 50% efficiency). Using the calculated R_0 and the estimated R values, the quenching efficiency (ϵ) by energy transfer can be calculated by the equation $\epsilon = R_0^6/(R_0^6 + R^6)$ (Förster, 1948). The ϵ values for Trp 309 and Trp 372 are calculated to be 82.2% and 27.8%, respectively.

The mechanism-based inhibitor (DHCaA) quenches the tryptophan fluorescence of AdoHcy hydrolase (NAD^+ form) by 35%, significantly greater than the 10% quenching observed in either the apo-AdoHcy hydrolase induced by the same inhibitor or the NAD^+ form of the enzyme induced by 3′-keto-Ado. Assuming that only Trp 309 and Trp 372 are responsible for the fluorescence quenching caused by energy transfer, the tryptophan fluorescence quenching of the enzyme induced by mechanism-based inhibitors is calculated to be 18.4% by using the above calculated efficiency values. This calculated quenching value is approximately the same as the experimentally measured quenching values (18–25%, based on the lifetime data or steady-state measurements). Coincident with the reduction of the enzyme-bound NAD^+ to NADH, there is a secondary conformational change, as indicated by the quenching constant (K_{sv}) by hydrophilic quencher (KI) that is larger in the NAD^+ form of the enzyme than in the apo form after binding with the mechanism-based inhibitor (DHCaA). In fact, this secondary conformational change may account for the tight binding between the mechanism-based inhibitor and the NAD^+ form of AdoHcy hydrolase. This observation may be the first evidence in support of the hypothesis proposed earlier (Abeles et al., 1982) that reduction of enzyme-bound NAD^+ to NADH may convert the enzyme to a "closed form" that traps the oxidized product.

Mechanism of AdoHcy Hydrolase (NAD^+ Form) Inactivation. The inactivation of AdoHcy hydrolase by mechanism-based inhibitors has been proposed to be a cofactor depletion mechanism (Wolfe & Borchardt, 1991), which is represented in the equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow[NAD^+ \quad NADH]{k_{inact}} E\text{–}I^* \qquad (2)$$

where k_{-1}/k_1 is the apparent reversible dissociation constant (K_i) of the enzyme–inhibitor complex (E·I) and k_{inact} is the rate constant for the irreversible inactivation. The present fluorescence quenching study makes this inactivation mechanism directly visible on the fluorescent screen and provides further support for the mechanism. The fluorscence quenching, because of the initial conformational change induced by ligand binding, may correspond to the reversible equilibrium step. The time-dependent quenching caused by further conformational change and energy transfer from tryptophan to NADH may correspond to the NAD^+ reduction step in eq 2. In fact, the fluorescence quenching data can be used to analyze the inhibition kinetic constants (K_i and k_{inact}) for mechanism-based inhibitors. If the data shown in Figure 2 for DHCaA are plotted as ln (% of $(1 - \Delta F/\Delta F_{max})$) *vs* time to obtain the pseudo-first-order rate constants (k_{app}), and these k_{app} values are plotted *vs* inhibitor concentration [I] as a double-reciprocal plot using the equation:

$$1/k_{app} = 1/k_{inact} + (K_i/k_{inact})/[I] \qquad (3)$$

then the K_i and k_{inact} for the enzyme inhibition by DHCaA are calculated to be 240 nM and 0.1 min^{-1}, respectively. These data are in good agreement with the data obtained by standard methodologies used to measure enzyme inhibition (K_i = 156 nM, k_{inact} = 0.09 min^{-1}) (Wolfe *et al.*, 1992).

Further studies using site-directed mutagenesis techniques to confirm these fluorescence quenching and enzyme inactivation mechanisms are currently ongoing in our laboratory.

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