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Immunoaffinity Purification and Fluorescence Studies of Human Adenosine Deaminase†

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Received October 15, 1986; Revised Manuscript Received December 18, 1986

ABSTRACT: Human thymus adenosine deaminase was isolated by using a monoclonal antibody affinity column. The highly purified enzyme produced by this rapid, efficient procedure had a molecular weight of 44 000. Quenching of the intrinsic protein fluorescence by small molecules was used to probe the accessibility of tryptophan residues in the enzyme and enzyme–inhibitor complexes. The fluorescence emission spectrum of human adenosine deaminase at 295-nm excitation had a maximum at about 335 nm and a quantum yield of 0.03. Addition of polar fluorescence quenchers, iodide and acrylamide, shifted the peak to the blue, and the hydrophobic quencher trichloroethanol shifted the peak to the red, indicating that the emission spectrum is heterogeneous. The fluorescence quenching parameters obtained for these quenchers reveal that the tryptophan environments in the protein are relatively hydrophobic. Binding of both ground-state and transition-state analogue inhibitors caused decreases in the fluorescence intensity of the enzyme, suggesting that one or more tryptophans may be near the active site. The kinetics of the fluorescence decrease were consistent with a slow conformational alteration in the transition-state inhibitor complexes. Fluorescence quenching experiments using polar and nonpolar quenchers were also carried out for the enzyme–inhibitor complexes. The quenching parameters for all enzyme–inhibitor complexes differed from those for the uncomplexed enzyme, suggesting that inhibitor binding causes changes in the conformation of adenosine deaminase. For comparison, parallel quenching studies were performed for calf adenosine deaminase in the absence and presence of inhibitors. While significant structural differences between adenosine deaminase from the two sources were evident, our data indicate that both enzymes undergo conformational changes on binding ground-state and transition-state inhibitors.

Adenosine deaminase (EC 3.5.4.4), a purine salvage pathway enzyme, catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. The enzyme is widely distributed in nature, and the highest activity in man is found in lymphoid tissues (Brady & O'Donovan, 1965). Adenosine deaminase is necessary for a functional immune system. An inherited deficiency of this enzyme in its most extreme form is associated with severe combined immunodeficiency disease (SCID;[1] Giblett et al., 1972). SCID is typically associated with severe lymphopenia as well as

†This work was supported by NIH Grants CA26391 and GM35009. D.J.R. was the recipient of USPHS National Research Service Award CA07524 from the National Cancer Institute.
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[1] Abbreviations: ADA, adenosine deaminase; ELISA, enzyme-linked immunoabsorbant assay; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; DHMPR, 1,6-dihydro-6-(hydroxymethyl)purine riboside; PAS, protein A–Sepharose CL-4B; PBS, phosphate-buffered saline; SCID, severe combined immunodeficiency disease; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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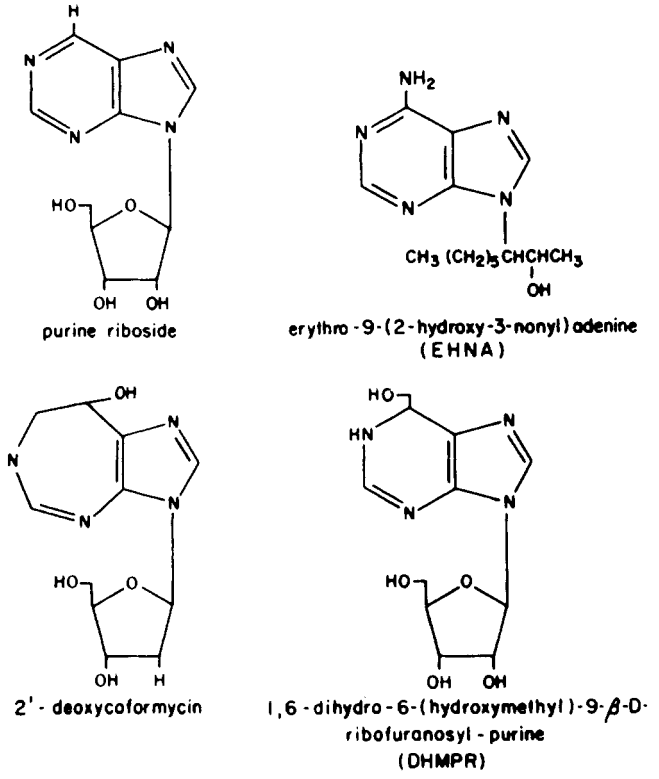

FIGURE 1: Structures of adenosine deaminase inhibitors.

abnormal lymphocyte function (Parkman et al., 1975; Agarwal et al., 1976; Cohen, 1975; Coleman et al., 1978). Overproduction of adenosine deaminase has also been observed in man and mouse. A marked increase in adenosine deaminase has been associated with certain leukemias (Hutton & Coleman, 1975; Smyth & Harrap, 1975) as well as a hereditary form of hemolytic anemia (Meier et al., 1976; Valentine et al., 1977). The association of hereditary deficiency of adenosine deaminase with selective destruction of lymphocytes created interest in the use of enzyme inhibitors for chemotherapy.

A number of substrate analogues with a wide range of binding affinities are competitive inhibitors of adenosine deaminase. These inhibitors are classified as ground or transition-state analogues. Ground-state analogue inhibitors have an aromatic purine similar to adenosine, and transition-state analogue inhibitors resemble the tetrahedral transition-state intermediate in the reaction catalyzed by adenosine deaminase. Ground-state inhibitors include purine riboside ($K_i \sim 10^{-6}$ M; Wolfenden et al., 1969; Frieden et al., 1980) and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA; $K_i = 1.6 \times 10^{-9}$ M; Agarwal et al., 1977). Transition-state inhibitors exhibit slow binding kinetics: 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR; $K_i \sim 10^{-6}$ M; Evans & Wolfenden, 1970) and the antibiotics of coformycin and deoxycoformycin ($K_i \sim 10^{-11}$ M; Agarwal et al., 1977) which are stoichiometric tight-binding inhibitors. The structures of four of these inhibitors are shown in Figure 1.

Adenosine deaminase has been isolated from human tissues in two major molecular weight forms. The small form of molecular weight 36 000–44 000 is catalytically active (Van der Weyden et al., 1976; Wiginton et al., 1981). The large form of molecular weight 298 000 is a complex of the small form and a noncatalytic binding protein of unknown function (Daddona, 1979). The enzyme is coded for by a single genetic locus on the long arm of chromosome 20 (Creagen et al., 1974; Tischfield et al., 1974). Human adenosine deaminase cDNA has been cloned, and its nucleotide sequence has been determined (Wiginton et al., 1984; Daddona et al., 1984). The amino acid sequence of the enzyme has been derived from the cDNA sequence as well as from the partial amino acid sequence of tryptic peptides (Daddona et al., 1984). The sequence data predict a molecular weight of 40 638.

The human enzyme has a K_m for the substrate adenosine in the micromolar range (Agarwal et al., 1975). Kinetic analyses were used to compare calf intestinal and human erythrocytic adenosine deaminases, and no qualitative differences in substrate and inhibitory activities were observed. However, the kinetic parameters of the two enzymes are slightly different (Agarwal et al., 1975). The K_m values for the substrates adenosine and deoxyadenosine are lower for human than calf adenosine deaminase. In addition, the rates of association of enzyme and inhibitors are up to 3-fold faster for human erythrocytic adenosine deaminase than for calf intestinal adenosine deaminase. It has been suggested that the lower K_m and K_i values observed with the human enzyme may result from these faster association rates (Agarwal et al., 1977). Frick et al. (1986) studied the binding of deoxycoformycin to calf adenosine deaminase to determine the forces stabilizing the enzyme–substrate complex in the transition state. No covalent bonds were formed on binding of the transition-state analogue. Previously, it had been shown that adenosine deaminase catalyzes the stereospecific addition of water to pteridine (Evans & Wolfenden, 1973). From these results, they concluded that adenosine deaminase mediates a direct water attack on its substrates.

Fluorescence spectroscopy has been used to probe the solution conformation of calf intestinal adenosine deaminase (Skorka et al., 1981; Kurz et al., 1985). Because the primary structure of the human adenosine deaminase is now known and the enzyme is clinically significant, we initiated physical studies of the human enzyme. A necessary first step was the development of a purification procedure suitable for small amounts of human tissue. We constructed an affinity column using monoclonal antibodies directed against human adenosine deaminase. The purification method based on this column was rapid and resulted in highly purified human enzyme in amounts sufficient for physical study. We then used the intrinsic protein fluorescence to determine the accessibility of tryptophans to external quenchers. The tryptophan environments in adenosine deaminase and in several enzyme–inhibitor complexes were examined by using polar and nonpolar quenchers. These studies indicated that the binding of both ground- and transition-state inhibitors causes conformational changes in the protein. Quenching studies were also carried out on calf intestinal adenosine deaminase. The results were compared to the data for the human enzyme and to the published data for the calf enzyme (Kurz et al., 1985).

Experimental Procedures

Materials

BALB/c mice were obtained from Harlan Sprague-Dawley (Madison, WI). Biochemical reagents for monoclonal antibody production and screening were identical with those previously described (Fuller et al., 1985). Immunoglobulin subtyping reagents were purchased from Boehringer Mannheim Biochemicals. Protein A–Sepharose CL-4B (PAS) was purchased from Sigma Chemical Co. [8-^{14}C]Adenosine was purchased from New England Nuclear Corp. Acrylamide was purchased from Bio-Rad Laboratories and was recrystallized from ethyl acetate for fluorescence quenching experiments. Ethylene glycol (99+%) and trichloroethanol were from Aldrich Chemical Co. Purine riboside was purchased from

Sigma Chemical Co., and EHNA was purchased from Burroughs Wellcome Co. (Research Triangle Park, NC). Deoxycoformycin was a gift from the National Cancer Institute. DHMPR was kindly provided by Dr. R. Wolfenden and was stored under nitrogen. A fluorescent impurity which forms in the DHMPR preparation upon exposure to air was removed by chromatography on a SiO_2 column under nitrogen using 30% (v/v) methanol in chloroform. Calf intestinal adenosine deaminase was purchased from Sigma Chemical Co. All other chemicals were reagent grade or higher. Phosphate-buffered saline (PBS) was 0.85 mM Na_2HPO_4, 0.18 mM NaH_2PO_4, and 0.14 M NaCl (pH 7.4).

Methods

Production of Monoclonal Antibodies. BALB/c mice were immunized with 0.5 μg of human adenosine deaminase given in multiple injections biweekly for 1–2 months. The antigen was purified from human lymphoblasts following the procedure of Wiginton et al. (1981) and was cross-linked with glutaraldehyde. Sera were tested periodically for the presence of antibodies by two methods: ELISA screening (Fuller et al., 1985) using 0.1 μg of protein/well and the immunoblot technique of Towbin et al. (1979) using 0.5 μg of protein/lane. Mice producing anti-adenosine deaminase antibodies (titers at 1:1000) were selected for cell fusion to produce hybridomas following the procedure of Oi and Herzenberg (1980). Established hybridoma cells were screened for production of antibodies by both ELISA and immunoblot procedures. Cells in wells that were positive for antibody production were cloned by limiting dilution; 12 positive clones were obtained and expanded. In addition, cells were injected into mice primed with 2,6,10,14-tetramethylpentadecane for the production of ascites fluid. The monoclonal antibodies were purified from culture media by affinity column chromatography using goat anti-mouse immunoglobulins (IgG + IgM + IgA) conjugated to CNBr-activated Sepharose 4B (Fuller et al., 1985) or from ascites fluid using a PAS column.

Characterization of Monoclonal Antibodies. The purified antibodies were analyzed by SDS–polyacrylamide gel electrophoresis and were identified as immunoglobulin G. The antibodies were subclassed by ELISA analysis using subtyping reagents.

The affinity of the antibodies for human adenosine deaminase was estimated from the amount of antibody required to bind 50% of enzyme activity. In this binding assay, the higher affinity antibodies bind at lower protein concentrations. Variable quantities of purified antibody from each clone were incubated overnight at 4 °C with lymphoblastoid cell extract containing 0.1 unit of activity. The antibody–antigen complexes were precipitated with PAS, and the supernatants were assayed for residual enzyme activity. Adenosine deaminase activity was assayed by monitoring the conversion of [^{14}C]-adenosine to [^{14}C]inosine using paper chromatography as previously described (Coleman & Hutton, 1975). One unit of adenosine deaminase activity is defined as the amount of enzyme required to produce 1 μmol of inosine/min at 35 °C.

Neutralization of enzyme activity with individual monoclonal antibodies was tested by adding 50 μL of media from each clone to lymphoblastoid cell extract containing 0.25 unit of enzyme. The media were assayed for enzyme activity after an overnight incubation at 4 °C. Individual monoclonal antibodies were tested for cross-reactivity with calf adenosine deaminase. These enzymes were blotted onto nitrocellulose paper following SDS–polyacrylamide gel electrophoresis. The nitrocellulose paper was incubated with concentrated hybridoma culture media from individual clones and was developed as described for the immunoblotting procedure.

Construction of Immunoaffinity Column. The monoclonal antibody with the highest affinity (5 μg) for adenosine deaminase (N1D1) was selected for the immunoaffinity matrix. The matrix was constructed by cross-linking the antibody to PAS using dimethyl suberimidate. PAS (1.5 g) was hydrated in 0.2 M triethanolamine adjusted to pH 8.7 with HCl and was washed extensively in the same buffer. The column material was resuspended in 10 mL of the buffer. The antibody source was usually a solution of ascites fluid diluted 1:4 in PBS. In some cases, antibody purified from culture media was used. Antibody was diluted to a final volume of 10 mL in PBS, and the pH was adjusted to 8.7 with triethanolamine. The antibody solution was added to the PAS suspension gradually with stirring and was allowed to mix for 1 h. Antibody binding to the PAS was monitored by estimating the amount of protein in the supernatant from the A_{280}/A_{260} ratio or from SDS–polyacrylamide gel electrophoresis. The PAS–monoclonal antibody suspension was centrifuged to remove unbound antibody and was resuspended to 10 mL of PBS. The antibody was cross-linked to PAS by gradually adding 30 mg of dimethyl suberimidate while stirring and gently mixing the suspension for 3–4 h. The PAS–monoclonal antibody suspension was poured into a 1 × 20 cm column and was washed extensively with the following solutions to remove any un-cross-linked antibody: PBS, 2 M $MgCl_2$, 2 M NaCl, and a low-pH buffer (50 mM glycine hydrochloride, pH 3.0, and 0.5 M NaCl). The column (about 5-mL bed volume) was stored at 4 °C in PBS containing 0.02% sodium azide.

Immunoaffinity Chromatography of Adenosine Deaminase. Human thymus tissue from thoracic surgery was used as the source of the enzyme. Usually 20–25 g of tissue was extracted at 4 °C in 4 volumes of PBS using a Sorvall Omnimixer. The extract was centrifuged at 35000 rpm (Ti45 rotor) for 40 min, and the supernatant was collected. The crude extract was incubated with the entire PAS–monoclonal antibody matrix (about 5 mL) overnight at 4 °C with gentle rotation. The rest of the purification procedure was carried out at room temperature. The suspension was transferred to a 1 × 20 cm column. The unbound fraction was collected and assayed for enzyme activity. The column was washed with 100 mL of PBS followed by 100 mL of 2 M KCl to remove nonspecifically bound proteins. The enzyme was eluted from the column using 50 mL of 4 M urea in PBS followed by 25 mL of 6 M urea in PBS. These two fractions were concentrated and dialyzed against PBS by ultrafiltration (PM-10 membrane). In some cases, the enzyme solution was passed through a second column of PAS to remove IgG that eluted with the enzyme. Enzyme purity was monitored by SDS–polyacrylamide gel electrophoresis and by immunoblot analysis. Enzyme activity was assayed as described above, and protein concentration was determined by the method of Lowry et al. (1951). The enzyme was concentrated to 1–2 mg/mL and stored in PBS containing 50% glycerol at −20 °C.

Fluorescence Measurements. Fluorescence was measured on a SPEX Fluorolog 211 controlled by a Datamate terminal or on a SLM 8000 photon-counting spectrofluorometer interfaced to an Apple II$^+$ microcomputer. Excitation was at 295 nm (to avoid excitation of tyrosine residues), and emission was measured between 300 and 400 nm. Fluorescence data were collected as a ratio of emission to lamp reference. Emission spectra were corrected for wavelength-dependent instrument response. All fluorescence measurements were made under "magic-angle" conditions to eliminate anisotropic

effects. For the SPEX fluorometer, the exciting light at 295 nm was unpolarized, and the emission polarizer was oriented at 35°. For the SLM fluorometer, the excitation and emission polarizers were set at 55° and 0°, respectively. Fluorescence experiments were done on 1 μM solutions of adenosine deaminase in PBS in 1-mL semi-microcuvettes at 25 °C. The experiments on enzyme–inhibitor complexes were performed in the presence of saturating concentrations of inhibitor: 2 μM EHNA, 100 μM purine riboside, 1–2 μM deoxycoformycin, and 20 μM DHMPR.

Quantum yields were measured relative to quinine sulfate. Fresh solutions of quinine bisulfate (Eastman Kodak) were prepared by dissolving a crystal in 1 N H_2SO_4 (double distilled, GFS Chemicals) and adjusting the absorbance to <0.1 at 345 nm. Sample quantum yields were calculated with $\phi = 0.546$ for quinine sulfate (Melhuish, 1961).

The kinetics of inhibitor binding to adenosine deaminase were monitored by the change in protein fluorescence. The adenosine deaminase solution was placed in the fluorometer in a 3-mL cuvette containing a magnetic stir bar. The fluorescence intensity was acquired at 338 nm for 1-s intervals. A small volume of inhibitor solution was added while stirring and acquiring intensity data. The mixing time of the apparatus (≃4 s) was estimated by adding *N*-acetyltryptophanamide solution to PBS while stirring, and determining the time required for the fluorescence intensity to reach a plateau.

Quenching experiments were carried out by using iodide, trichloroethanol, and acrylamide. Stock solutions of 1 M KI in water (containing a trace of thiosulfate to retard I_3^- formation), 1 M trichloroethanol in ethylene glycol, and 1, 2, and 8 M acrylamide in water were prepared. The ionic strength was kept constant for iodide quenching experiments by diluting adenosine deaminase into PBS containing 1 M KCl. The fluorescence intensity was measured as a function of quencher concentration at 338-nm emission wavelength. At each quencher concentration, intensities were acquired for three 5-s intervals, and the values were averaged. Intensities from blanks containing buffer and quencher were acquired in the same manner and subtracted from sample intensities. The activity of the enzyme was assayed in the presence of the highest quencher concentration. No changes in enzyme activity were observed in 1 M KI, 300 mM trichloroethanol, or 1 M acrylamide. A nonfluorescent impurity present in purine riboside was suspected of affecting the experimental results. Removal of this impurity by HPLC purification had no effect on the data. To correct for residual fluorescent impurity in DHMPR (about 10% of adenosine deaminase intensity at 338 nm), which does not bind to the enzyme (Kurz et al., 1985), the blank also contained DHMPR. In most cases, the absorbance at 295 nm was less than 0.1. The correction for inner filter effects was applied to samples containing DHMPR and high concentrations of acrylamide and was less than 1.5 (Lakowicz, 1983).

The quenching data were analyzed by using the Stern–Volmer equation

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

or the modified Stern–Volmer equation

$$F_0/\Delta F = 1/f_a + 1/f_a K_{sv}[Q] \quad (2)$$

where F_0 is the fluorescence in the absence of quencher, F is the fluorescence in the presence of quencher, $\Delta F = F_0 - F$, [Q] is the concentration of quencher, f_a is the fraction of total emission accessible to the quencher, and K_{sv} is the Stern–Volmer constant. The quenching parameters K_{sv} or K_{sv} and f_a were determined by linear regression of the Stern–Volmer plots of F_0/F vs. [Q] or the modified Stern–Volmer plots of $F_0/\Delta F$ vs. $1/[Q]$. Three experiments were performed for trichloroethanol and acrylamide quenching, and one or two experiments were performed for iodide quenching. Mean values of the quenching parameters from multiple experiments are reported. In all cases, errors in K_{sv} and f_a were less than 10%. The errors in the mean values were standard deviations. The errors in single values were propagated uncertainties in the slope and intercept (Bevington, 1969).

Table I: Characteristics of Monoclonal Antibodies Directed against Human Adenosine Deaminase

hybridoma cell line	binding assay (μg)[a]	immunoglobulin subclass	neutralization[b]	cross-reactivity to calf ADA
group I				
N1D1	5	IgG 1, κ	–	–
Q1B2	13	IgG 1, λ	–	–
R5A5	18	IgG 1, λ	–	–
group II				
R6B2	30	IgG 2b, λ	–	–
R5C5	35	IgG 1, λ	–	–
R2D2	35	IgG 1, λ	–	+
Q2A6	44	IgG 1, λ	–	–
N2A5	46	IgG 1, λ	+	–
R4C1	53	IgG 1, λ	–	+
group III				
R4D1	95	IgG 1, λ	–	–
R2C3	109	IgG 1, λ	–	–
N1C1	189	IgG 1, λ	–	–

[a] Monoclonal antibody binding activity was estimated from the quantity of antibody required to precipitate 0.5 unit of enzyme activity in the presence of PAS. [b] Neutralization of enzyme activity was tested by incubating 50 μL of hybridoma culture medium with 0.25 unit of adenosine deaminase. Residual enzyme activity was assayed following an overnight incubation of 4 °C.

Results

Characterization of Monoclonal Antibodies. Twelve monoclonal cell lines producing antibody to human adenosine deaminase were developed. Epitope specificities were not determined. However, the antibodies were classified roughly into three groups on the basis of their relative affinities for adenosine deaminase. These groups are labeled I, II, and III for high, moderate, and low affinities, respectively (Table I). The immunoglobulin classes of these antibodies were determined to be mainly IgG 1 with one IgG 2b. The light chains of all the antibodies were λ with the exception of one (N1D1) which was κ.

The antibodies were further characterized by assessing enzyme neutralization as well as cross-reaction with calf intestinal adenosine deaminase. Only two of the clones produced antibody that cross-reacted with the calf enzyme. The one antibody that neutralized the activity of human adenosine deaminase (N2A5) did not cross-react with calf adenosine deaminase. However, it is unlikely that the active sites of the two enzymes are different since both enzymes have similar K_m and K_i values for the same substrates and inhibitors (Agarwal et al., 1975). It is more probable that the N2A5 antibody binds at a site near the active site of the human enzyme and prevents the substrate from reaching the active site. This particular antibody binding site must be absent in the calf enzyme. The fact that only two of the clones produced antibodies that cross-reacted with the calf enzyme suggests that there may be significant differences in the primary structures of calf and human adenosine deaminases.

Purification of Adenosine Deaminase by Immunoaffinity Chromatography. Several different procedures were inves-

Table II: Single Purification of Human Thymus Adenosine Deaminase by Monoclonal Antibody Affinity Column

	volume (mL)	total act. (units)[a]	total protein (mg)	% act. recovered	sp act. (units/mg)
crude extract[b]	100	428	410	100	1.04
purified protein	0.5	200	0.6	47	333

[a] A unit of adenosine deaminase is defined as the amount required to deaminate 1 μmol of adenosine/min at 35 °C. [b] This preparation contained 21 g of thymus tissue.

tigated in the construction of an affinity matrix for adenosine deaminase using the monoclonal antibodies. Antibody from the clone N1D1, which had the highest affinity for adenosine deaminase, was initially immobilized on derivatized controlled-pore glass beads following the procedure of Fuller et al. (1985), but the matrix exhibited low binding efficiency of the enzyme from crude extract. The attachment of antibodies in random orientations on this matrix apparently decreases the availability of antigen binding regions. A second procedure attached the antibody to Sepharose beads through a biotin–avidin linkage (Fuller et al., 1985). This matrix also exhibited low binding efficiency of the enzyme, and the antibody leached continuously from the Sepharose. The most efficient binding of adenosine deaminase from crude extract was achieved by immobilization of the antibody on PAS. The specific binding of antibody to this solid support through the F_c portion of the immunoglobulins leaves the antigen binding site free to react with the enzyme, and the chemical cross-linkage prevents the antibody from washing off the column. Antibodies from other clones with lower affinity for adenosine deaminase were less efficient at retaining the enzyme on the column. Even when combinations of these antibodies were used, no additive effects were obtained.

Elution of active enzyme was achieved with 4 M urea. A second elution using 6 M urea was usually necessary to recover additional enzyme. Higher concentrations of urea did not result in further recovery. Elution with salt solutions, such as 1 and 2 M $MgCl_2$ and $CaCl_2$, and low-pH buffers inactivated the enzyme. Table II summarizes the results from one purification. Elution with 4 M urea yielded 80% of the purified enzyme activity; 10–20% additional activity was obtained with a final wash using 6 M urea. Yields as high as 50% of starting activity have been obtained though the average yields were about 40–45%. The specific activity of the purified thymus enzyme ranged from 330 to 425 units/mg. The samples eluted from the column were analyzed on an SDS–polyacrylamide gel to assess purity, and the results of a typical experiment are shown in Figure 2. The purified enzyme sample occasionally exhibited two minor bands (<5% of total protein) in addition to the major adenosine deaminase band at 44 000 molecular weight. These minor bands were found to cross-react with both polyclonal and monoclonal antibodies to human adenosine deaminase by immunoblot analysis. They probably represent proteolytic degradation products of the enzyme.

The error in replicate enzyme assays in our laboratory with highly purified adenosine deaminase is about 25% (±100 units). Human adenosine deaminase has also been purified from granulocytes, erythrocytes, and kidney tissue. The specific activities obtained for those purified enzymes (with the activities corrected to 35 °C) were 486 units/mg for granulocytes (Wiginton et al., 1981), 412–430 units/mg for erythrocytes (Schrader et al., 1976; Daddona & Kelly, 1977), and 53 units/mg for kidney (Schrader & Stacy, 1977). Thus, our range of enzyme activity for human thymus is comparable

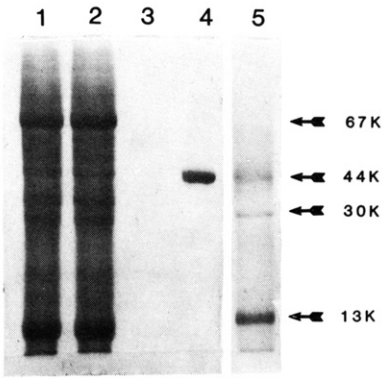

FIGURE 2: SDS–polyacrylamide gel electrophoresis of human adenosine deaminase purified by immunoaffinity chromatography. Lane 1, crude thymus preparation; lane 2, run-through from column; lane 3, PBS wash; lane 4, pure adenosine deaminase; lane 5, molecular weight markers.

to our published data for granulocytes and to activities reported by others for erythrocytes. Moreover, different adenosine deaminase preparations from thymus show identical fluorescence parameters in the presence and absence of inhibitors, suggesting that the protein is fully active following immunoaffinity purification. The specific activity for pure kidney adenosine deaminase is much lower. As indicated earlier, the molecular weights reported for human adenosine deaminase from SDS–polyacrylamide gel electrophoresis range from 36 000 to 44 000, while the human cDNA codes for a molecular weight of 40 638. It is not known whether the apparent molecular weight variation is real or whether it is due to differences in the standards used on the gels or perhaps to tissue-specific posttranslational modifications that affect migration.

The monoclonal antibody column provides a rapid one-step procedure for the purification of milligram quantities of highly purified adenosine deaminase. This procedure has distinct advantages over the procedure of Wiginton et al. (1981) previously used in our laboratory. It takes about 2 days compared to 2 weeks for the conventional procedure. Since the procedure is relatively rapid, it can be carried out at room temperature instead of 4 °C with no deleterious effects. Finally, this procedure can be readily adapted to quantities of starting material ranging from 1 to 500 g of tissue, with no difference in final yield.

Tryptophan Fluorescence of Human Adenosine Deaminase. Human adenosine deaminase has four tryptophan residues in the predicted amino acid sequence (Wiginton et al., 1984). Daddona et al. (1984) determined the partial amino acid sequence from tryptic peptides, which confirms three of the four tryptophans. These residues are probably in different environments in the folded protein, and their accessibilities to small molecules that quench tryptophan fluorescence might be different. In order to probe the tryptophan environments in adenosine deaminase, we selected fluorescence quenchers which preferentially reach ionic and hydrophobic regions in proteins: iodide and trichloroethanol. Since iodide is an ionic quencher, it normally quenches the fluorescence of surface tryptophans, whereas trichloroethanol senses residues in hydrophobic regions of proteins and often causes exaggerated quenching (Eftink et al., 1977). Acrylamide, a neutral quencher which readily quenches the fluorescence of solvent-accessible residues in polar environments, was included for comparison.

The emission spectrum of adenosine deaminase has a peak at about 335 nm as shown in Figure 3. Tryptophan fluorescence is sensitive to solvent polarity, having an emission

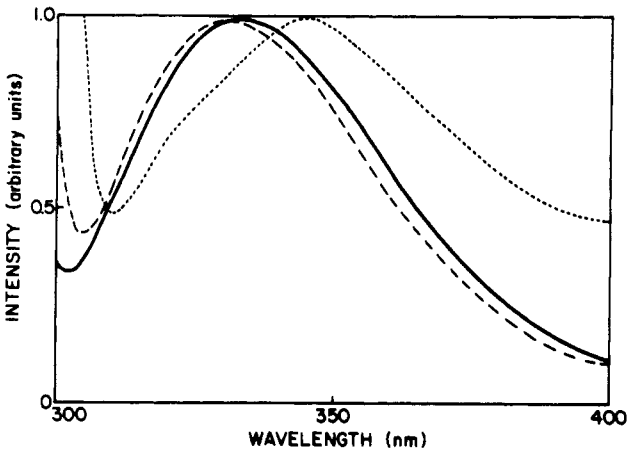

FIGURE 3: Fluorescence emission spectra of adenosine deaminase in the absence (—) and presence of quenchers: (- - -) 1 M acrylamide or 1 M iodide and (···) 300 mM trichloroethanol. Samples were excited at 295 nm, and the spectra were normalized at their peaks.

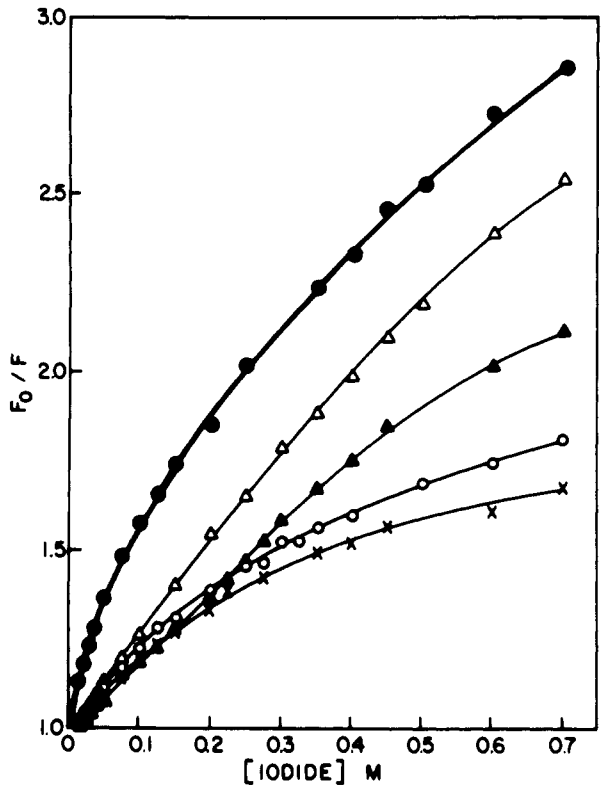

FIGURE 4: Stern–Volmer plots of adenosine deaminase (●) and adenosine deaminase–inhibitor complexes quenched by iodide: (△) 2 μM EHNA, (○) 100 μM purine riboside, (▲) 1–2 μM deoxycoformycin, and (×) 20 μM DHMPR. Adenosine deaminase concentration was 1 μM.

maximum at 300 nm in hexane (Lakowicz, 1983) and at 348 nm in water (Teale & Weber, 1957). In multi-tryptophan proteins, selective quenching of tryptophans in polar or nonpolar environments by small-molecule quenchers will cause shifts in the position of the emission maximum. As shown in Figure 3, iodide and acrylamide (1 M) shifted the peak to shorter wavelengths by about 3 nm, whereas trichloroethanol (300 mM) shifted the peak to longer wavelengths by about 7 nm. These spectral shifts suggest that the emission spectrum of adenosine deaminase is comprised of heterogeneous emission from tryptophans in different environments. Because the enzyme is fully active at the maximum concentration of quencher, these shifts cannot be due to protein conformational changes that affect enzyme function.

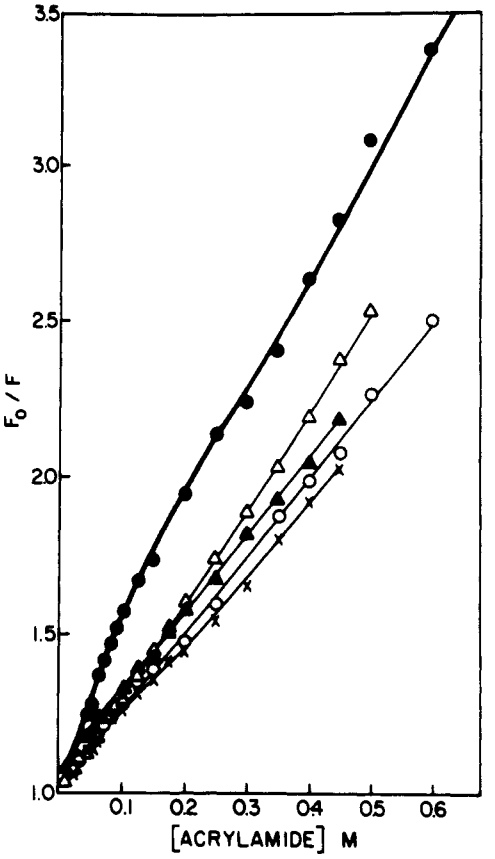

FIGURE 5: Stern–Volmer plots of adenosine deaminase (●) and adenosine deaminase–inhibitor complexes quenched by acrylamide: (△) 2 μM EHNA, (○) 100 μM purine riboside, (▲) 1–2 μM deoxycoformycin, and (×) 20 μM DHMPR. Adenosine deaminase concentration was 1 μM.

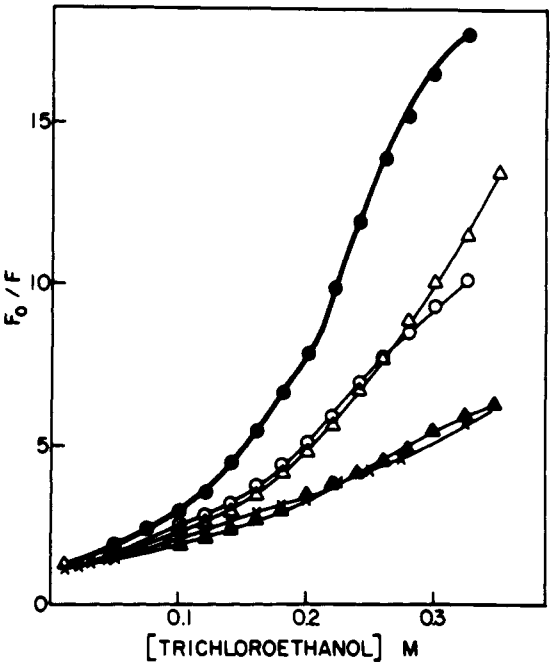

FIGURE 6: Stern–Volmer plots of adenosine deaminase (●) and adenosine deaminase–inhibitor complexes quenched by trichloroethanol: (△) 2 μM EHNA, (○) 100 μM purine riboside, (▲) 1–2 μM deoxycoformycin, and (×) 20 μM DHMPR. Adenosine deaminase concentration was 1 μM.

Stern–Volmer plots for iodide, trichloroethanol, and acrylamide quenching of adenosine deaminase emission are depicted by the heavy lines in Figures 4–6. The Stern–Volmer plot for iodide showed downward curvature. This is commonly

Table III: Fluorescence Quenching Parameters from Modified Stern–Volmer Plots[a]

		human ADA			calf ADA		
quencher	inhibitor	[Q] (mM)	K_{sv} (M^{-1})	f_a	[Q] (mM)	K_{sv} (M^{-1})	f_a
iodide	none	0–50	8.5	0.47	0–50	8.1	0.27
		100–700	5.4	0.80	100–700	2.4	0.59
	EHNA	50–700	2.6	0.96	50–700	1.7	0.55
	purine riboside	50–700	3.9	0.55	50–700	1.8	0.51
	deoxycoformycin	50–700	5.5	0.62	50–700	2.1	0.45
	DHMPR	50–700	5.4	0.50	50–700	2.5	0.32
acrylamide	none	50–100	14.5	0.55	50–700	3.4	0.97
		125–700	8.1	0.81			
	EHNA	50–700	2.9	1.03	50–700	2.6	0.98
	purine riboside	50–700	3.0	0.92	50–700	2.9	1.02
	deoxycoformycin	50–700	3.3	0.92	50–700	2.3	0.95
	DHMPR	50–700	2.7	0.92	50–700	1.3	1.03

[a] Adenosine deaminase concentration was 1 μM. Saturating concentrations of inhibitor were 2 μM EHNA, 100 μM purine riboside, 1–2 μM deoxycoformycin, and 10 μM DHMPR.

Table IV: Fluorescence Quenching Parameters from Stern–Volmer Plots[a]

			K_{sv}(eff) (M^{-1})[b]	
quencher	inhibitor	[Q] (mM)	human ADA	calf ADA
trichloroethanol	none	0–140	22.0	28.3
	EHNA	0–140	13.6	18.8
	purine riboside	0–140	16.5	21.1
	deoxyco-formycin	0–140	10.2	13.7
	DHMPR	0–140	11.7	6.1

[a] Adenosine deaminase concentration was 1 μM. Saturating concentrations of inhibitor were 2 μM EHNA, 100 μM purine riboside, 1–2 μM deoxycoformycin, and 10 μM DHMPR. [b] $K_{sv}(\text{eff}) = \sum f_i K_{sv_i}$ is the effective Stern–Volmer quenching constant as described in the text.

Table V: Decrease of Intrinsic Fluorescence of Adenosine Deaminase by Inhibitors[a]

	$\Delta F/F_0$ (%)[b]	
inhibitor	human ADA	calf ADA
EHNA	0–2	0–2
purine riboside	8–10	8–10
deoxycoformycin	30	35
DHMPR	45	50

[a] Adenosine deaminase concentration was 1 μM. Saturating concentrations of inhibitor were 2 μM EHNA, 100 μM purine riboside, 1–2 μM deoxycoformycin, and 10 μM DHMPR. [b] Emission at 338 nm.

seen in proteins containing more than one class of tryptophans and suggests that a fraction of the total emission is not accessible to the quencher (Eftink & Ghiron, 1981). The modified Stern–Volmer plot was also nonlinear (not shown). The quenching parameters f_a and K_{sv} given in Table III were determined by fitting the linear regions of the modified plot to eq 2. Only 47% of the emission was accessible at low concentrations of iodide, whereas 80% of the emission became accessible at higher concentrations of iodide with a lower apparent K_{sv}. The 47% of the emission that was quenched by low concentrations of iodide with a higher K_{sv} probably represents surface tryptophans. The additional 30% of the emission that was accessible only at higher concentrations of iodide may be from surface residues in a less polar environment or from residues located in a cavity accessible to solvent. Similar results were obtained with the neutral, polar quencher acrylamide, but the K_{sv} values were somewhat higher (Table III). In contrast, trichloroethanol quenching of adenosine deaminase emission yielded a Stern–Volmer plot with steep upward curvature (Figure 6). This behavior is indicative of static quenching, a phenomenon that occurs when quencher and chromophore are so close together that the quenching occurs almost instantaneously (Eftink et al., 1977). The effective quenching constant, K_{sv}(eff), was calculated from the initial slope of the Stern–Volmer plot (Eftink & Ghiron, 1976), and the results are shown in Table IV. Since about 20% of the emission was resistant to quenching by both iodide and acrylamide, it appears that adenosine deaminase has at least one buried tryptophan.

In the case of multi-tryptophan proteins, the apparent quenching constant derived from a Stern–Volmer or modified Stern–Volmer analysis is a weighted function of the quenching constants of the individual tryptophans. Only at low quencher concentrations does this function assume a tractable form, where $K_{sv}(\text{eff}) = \sum f_i K_{sv_i}$ is the slope of the Stern–Volmer plot or the inverse slope ($f_a K_{sv}$) of the modified plot (Lehrer, 1971). Here, f_i is the fractional fluorescence intensity of residue i, which is proportional to its fluorescence lifetime(s) (τ_i), and K_{sv_i} is the quenching constant of residue i, which includes contributions from collisional (k_{q_i}) and static (V_i) quenching constants (Lehrer, 1971; Eftink & Ghiron, 1981). Despite this complexity, it is obvious that differences in the apparent quenching constants for efficient quenchers of indole fluorescence, such as iodide, acrylamide, and trichloroethanol (Eftink & Ghiron, 1981), reflect differences in the bimolecular k_{q_i} or static V_i quenching constants of one or more tryptophans. The data in Tables III and IV indicate that adenosine deaminase fluorescence is much more susceptible to quenching by low concentrations of nonpolar than polar quenchers: K_{sv}(eff) for trichloroethanol was about 3–5-fold greater than the values for acrylamide and iodide. This suggests that some tryptophan residues in adenosine deaminase are in a hydrophobic environment. Moreover, since the fraction of the emission accessible to iodide and acrylamide was about the same, it appears that the polar quenchers are reaching the same tryptophans. In this case, the lower K_{sv} values for iodide compared to acrylamide would be consistent with a relatively hydrophobic environment of these residues.

In the presence of inhibitors, the intrinsic fluorescence of adenosine deaminase was decreased 0, 10, 30, and 45% for EHNA, purine riboside, deoxycoformycin, and DHMPR, respectively, with no shift in the emission maximum (Table V). There are three possible interpretations for the decrease in fluorescence intensity upon complex formation: (1) tryptophan may transfer energy to the inhibitor; (2) at least one tryptophan residue may be near the active site of the enzyme whose emission is quenched in the complex; and (3) a conformational change may occur in the protein which quenches the emission of tryptophans remote from the active site. Time-resolved fluorescence measurements show that the

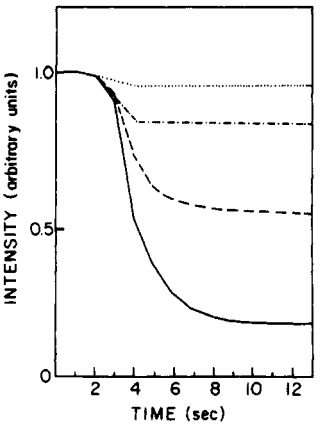

FIGURE 7: Kinetics of decrease in fluorescence of 1 μM adenosine deaminase on addition of inhibitor: (···) 2 μM EHNA, (-·-) 100 μM purine riboside, (--) 2 μM deoxycoformycin, and (—) 20 μM DHMPR. The initial 4 s represents the mixing time of the apparatus. Intensities were not corrected for dilution.

fluorescence lifetimes of adenosine deaminase are not affected by purine riboside and deoxycoformycin (Philips et al., 1986), ruling out energy transfer. Proximity of one or more tryptophans to the catalytic site is a plausible explanation. The finding that EHNA does not cause a decrease in protein fluorescence may reflect its alternate mode of binding. Woo and Baker (1982) have shown that the *erythro*-nonyl moiety of EHNA binds to the enzyme at an auxiliary region referred to as the "EHNA-specific region". This binding is believed to orient the purine moiety and the C-6 amino group away from the enzyme active site. Hence, the fact that the other three inhibitors decreased adenosine deaminase fluorescence suggests that there may be tryptophan residues near the active site whose fluorescence is quenched when the heterocyclic ring is positioned in the site. A conformational change in adenosine deaminase is apparent in the binding kinetics of deoxycoformycin and DHMPR monitored by fluorescence. The time-dependent decreases in adenosine deaminase fluorescence upon binding of purine riboside, deoxycoformycin, and DHMPR are presented in Figure 7. Purine riboside caused a single fast decrease in fluorescence corresponding to the 4-s mixing time of the apparatus, after which the intensity reached a plateau. In the case of the transition-state analogues deoxycoformycin and DHMPR, the decrease in fluorescence appeared to be biphasic. The initial rapid drop in intensity after mixing was followed by a slower decline. The larger decreases in intensity observed for deoxycoformycin and DHMPR (30% and 45%) compared to purine riboside (10%) as well as the biphasic intensity change upon binding of deoxycoformycin and DHMPR must be due to conformational changes in the transition-state inhibitor complexes.

Fluorescence Quenching of Enzyme–Inhibitor Complexes. The possibility that inhibitor binding induces conformational changes in adenosine deaminase was pursued by fluorescence quenching experiments. Changes in protein conformation which alter the accessibility of tryptophans to small-molecule fluorescence quenchers are expected to cause differences in the quenching parameters f_a and K_{sv} of enzyme–inhibitor complexes compared to enzyme alone. However, two other factors could also lead to differences in the quenching parameters. First, inhibitor binding may sterically block the access of quenchers to tryptophans near the active site. Second, the populations of fluorescent tryptophan residues may not be the same in the enzyme–inhibitor complex and in the uncomplexed enzyme. As mentioned before, the fluorescence lifetimes of enzyme–inhibitor complexes do not differ significantly from those of the enzyme alone. This suggests that the fluorescence decreases observed in the presence of inhibitor are mainly due to static quenching of tryptophan emission. Thus, part of the emission of the uncomplexed enzyme would not be available for quenching in the enzyme–inhibitor complex. The quencher would then only affect the remaining fluorescent tryptophans, which might have different quenching parameters. More complicated scenarios for redistributing the emission among the various tryptophans in the enzyme–inhibitor complex are also possible.

Binding of EHNA to adenosine deaminase does not change the intrinsic protein fluorescence. Thus, we infer that the emission available for quenching is the same in the absence and presence of EHNA. Any differences observed in the quenching parameters for the enzyme and the enzyme–inhibitor complex may be attributed to conformational changes in the protein when it binds EHNA or to steric effects. The Stern–Volmer plots for the enzyme–EHNA complex are shown in Figures 4–6. For iodide quenching, the curve had a lower slope than that found for enzyme alone, indicating a lower quenching rate. Acrylamide quenching gave a Stern–Volmer plot that was essentially linear, which means that all of the emission became accessible to acrylamide in the enzyme–EHNA complex. Trichloroethanol quenching yielded a Stern–Volmer plot with less steep upward curvature than that obtained for enzyme alone. The quenching parameters for the adenosine deaminase–EHNA complex are given in Tables III and IV. The values for all three quenchers changed upon binding of the inhibitor. The fractional accessibility, f_a, increased to almost 100% for both iodide and acrylamide quenching. This indicates that the previously buried tryptophan emission in adenosine deaminase has become accessible to the polar quenchers in the enzyme–inhibitor complex, arguing for a conformational change in the protein and against purely steric effects. The apparent K_{sv} as well as K_{sv}(eff) values decreased for all three quenchers. The about 2–3-fold lower values of K_{sv} for iodide and acrylamide undoubtedly reflect decreases in at least some of the bimolecular quenching constants k_q, implying that the environments of previously accessible tryptophans are less readily penetrated by quenchers in the enzyme–EHNA complex. Reductions in the extent of static quenching by both polar and nonpolar quenchers may have also occurred, as evidenced by the trichloroethanol quenching data. Overall, the tryptophan emission in the complex became more resistant to dynamic and static quenching processes. It therefore appears that binding of the ground-state inhibitor EHNA to adenosine deaminase caused a conformational change in the protein.

Binding of purine riboside, deoxycoformycin, and DHMPR decreases adenosine deaminase fluorescence by 10, 30, and 45%, respectively. In these cases, interpretation of quenching data is complicated by the fact that the fractional contributions of various tryptophan residues to the fluorescence emission differ for each enzyme–inhibitor complex and for the uncomplexed enzyme. Since the fluorescence lifetimes of adenosine deaminase do not change appreciably in the presence of inhibitor (Philips et al., 1986), we may assume that the decreased intensities of the enzyme–inhibitor complexes are due to static quenching of part of the protein fluorescence. The emission still available for quenching in the complexes would represent different subfractions of the total emission of the enzyme. Changes in the fractional accessibility, f_a, beyond those attributable to the decreased intensity could be indicative of conformational changes in the protein or steric effects. However, changes in the apparent quenching constants, K_{sv}

and K_{sv}(eff), are less readily interpreted. Even in the absence of conformational changes or steric effects in the enzyme–inhibitor complexes, the values of K_{sv} could change if the individual tryptophans have different K_{sv_i} values. Therefore, it is not surprising that the Stern–Volmer plots for the complexes with purine riboside, deoxycoformycin, and DHMPR shown in Figures 4–6 differ somewhat from each other as well as from the plots for the enzyme–EHNA complex and enzyme alone. Nevertheless, they share some common features, which are also evident in the quenching parameters given in Tables III and IV. Despite variations in the amount of fluorescence available for quenching, these enzyme–inhibitor complexes also appear to be more resistant to both polar and nonpolar quenchers than adenosine deaminase.

For iodide quenching, the Stern–Volmer plots curved downward (Figure 4), but the modified plots were linear (not shown). The values of f_a were in the range of 50–60% for all three enzyme–inhibitor complexes, compared to 80% for the uncomplexed enzyme (Table III). If the only effect of inhibitor binding were to decrease the intensities of emission from tryptophans accessible to iodide, the f_a values for the remaining emission should drop to 78, 71, and 67% for purine riboside, deoxycoformycin, and DHMPR, respectively. The significantly lower values obtained for f_a suggest a conformational change or steric effects. The latter is not necessarily inconsistent with the f_a value of 97% for the enzyme–EHNA complex, since EHNA purportedly binds to a different region of the active site. The apparent quenching constant K_{sv} for the purine riboside complex was lower than for enzyme alone, whereas the K_{sv} values for deoxycoformycin and DHMPR complexes were about the same as the value for the enzyme at higher iodide concentrations. In the case of acrylamide quenching, the Stern–Volmer plots for the three enzyme–inhibitor complexes were almost linear (Figure 5). The f_a and K_{sv} values were similar to the values obtained for the enzyme–EHNA complex (Table III). The slight increase in f_a to 92% implies that inhibitor binding decreased the intensity of tryptophans which were inaccessible to acrylamide in the uncomplexed enzyme or that a conformational change occurred. Since there is clear evidence for a conformational change in the EHNA complex and the quenching parameters for the other complexes are similar, we favor the latter interpretation. The quenching data for adenosine deaminase and the enzyme–EHNA complex suggest that the same tryptophans are accessible to both iodide and acrylamide. However, in the other enzyme–inhibitor complexes, 30–40% of the acrylamide-accessible emission was inaccessible to iodide, regardless of the amount of fluorescence available for quenching. While this might appear to invalidate some of our assumptions, we offer two plausible explanations in line with the above reasoning. First, binding of inhibitor may perturb the ionic environment of tryptophans near the active site, making them resistant to iodide but not acrylamide quenching. Second, the conformational change in the EHNA complex may be different from the changes in the other three complexes. The finding that the emission of the purine riboside, deoxycoformycin, and DHMPR complexes is slightly less accessible to acrylamide than the emission of the EHNA complex would be consistent with both conjectures. All of the enzyme–inhibitor complexes appear to be less susceptible to static quenching by trichloroethanol than the enzyme alone (Figure 6). The Stern–Volmer plots for the complexes with ground-state analogues, EHNA and purine riboside, were similar. Both plots exhibit less steep upward curvature than the plot for enzyme alone. Since the intensity decrease in the enzyme–purine riboside complex is only 10%, it is reasonable to attribute the reduction in static quenching in the two complexes to a common cause: a protein conformational change or possibly steric effects. The Stern–Volmer plots for the complexes with transition-state analogues, deoxycoformycin and DHMPR, were identical and show even less evidence of static quenching. The dramatic difference in the extent of static quenching by trichloroethanol for ground-state and transition-state inhibitor complexes supports the notion that the two types of inhibitors may induce different conformational changes in the protein. Alternatively, it may merely signify that the emission which remains available for quenching in the transition-state complexes represents tryptohan residues in polar environments less susceptible to static quenching. However, in this circumstance, the K_{sv}(eff) values might be expected to decrease more for trichloroethanol than for acrylamide quenching, which is not observed (Tables III and IV).

For those inhibitors that decrease intrinsic protein fluorescence, definitive conclusions cannot be derived from steady-state fluorescence quenching data. Even with this limitation, the results obtained indicate that binding of inhibitor renders the tryptophan emission of adenosine deaminase less accessible to small-molecule quenchers, probably by causing conformational changes in the protein.

Discussion

As a prelude to physical studies of human adenosine deaminase, we devised a simple, rapid procedure for protein purification. We produced monoclonal antibodies and constructed an immunoaffinity column. More than 80% of the antibodies were species specific for the human enzyme, and only one exhibited high enough binding affinity for efficient column chromatography. The anti-adenosine deaminase antibody cross-linked to PAS yielded a stable matrix that could be used to purify variable quantities of the human enzyme. The enzyme obtained exhibits a single band on SDS–polyacrylamide gel electrophoresis and has a high specific activity consistent with other highly purified enzyme preparations.

The high degree of species specificity of the monoclonal antibodies directed against human adenosine deaminase indicates that there are substantial differences in the primary structures of the human and calf enzymes. The human enzyme contains four tryptophans (Wiginton et al., 1984; Daddona et al., 1984). The amino acid sequence of calf adenosine deaminase has not been determined, but amino acid composition data suggest three tryptophans (Phelan et al., 1970). The fluorescence quantum yield of the calf enzyme is about 3 times higher than that of the human enzyme, 0.10 compared to 0.03, indicating that the tryptophan environments in the enzyme must differ.

Although the fluorescence quenching parameters of multi-tryptophan proteins cannot be interpreted in detail, useful information concerning the environment of tryptophan residues can often be obtained. We examined the solvent exposure of the tryptophans by using three small-molecule fluorescence quenchers of different polarity: iodide, acrylamide, and trichloroethanol. While this work was in progress, a report on acrylamide quenching of tryptophan emission in calf intestinal adenosine deaminase appeared (Kurz et al., 1985). Since our results for the human enzyme differed, we did quenching studies of the calf enzyme for comparison under our experimental conditions. Like human adenosine deaminse, the apparent Stern–Volmer constants, K_{sv}'s (Tables III and IV), for calf adenosine deaminase were lowest for iodide and highest for trichloroethanol quenching, indicating that the tryptophans

are in relatively hydrophobic environments. The K_{sv} for iodide quenching was about 2-fold lower, and the K_{sv}(eff) for trichloroethanol quenching was 1.3-fold higher than the values obtained for the human enzyme. This implies that the tryptophan environments are even more hydrophobic in the calf enzyme. The fraction of accessible emission (f_a) was lower for iodide than for acrylamide, suggesting that the environments of some tryptophans in the calf enzyme are negatively charged or highly hydrophobic.

The competitive inhibitors of adenosine deaminase have been classified into three kinetic groups (Frieden et al., 1980): (1) those that inhibit instantaneously (purine riboside); (2) those that inhibit rapidly but may cause a slow conformational change (EHNA); and (3) those that inhibit slowly (deoxycoformycin and DHMPR). The mechanisms of inhibition are postulated to be quite different for the three inhibitor classes. Purine riboside inhibits the enzyme like a classical competitive inhibitor. EHNA with its hydrophobic nonyl side chain binds competitively but incorrectly at the active site, and the resulting enzyme–inhibitor complex undergoes a rearrangement. DHMPR and deoxycoformycin are characterized by an apparent slow association rate constant for the formation of enzyme–inhibitor complex. This slow complex formation may reflect weak initial binding followed by a protein conformational change. These latter two transition-state inhibitors contain purine analogues that approximate the tetrahedral intermediate postulated in the direct-displacement reaction mechanism for the hydrolytic deamination of adenosine (Evans & Wolfenden, 1970; Frick et al., 1986). Binding of purine riboside, deoxycoformycin, and DHMPR decreases the intrinsic fluorescence of both calf and human adenosine deaminases, suggesting that there may be one or more tryptophan residues near the active site. The observation that EHNA did not affect the fluorescence is consistent with the hypothesis of Woo and Baker (1982) that EHNA does not bind directly at the enzyme active site. The ground-state inhibitor purine riboside caused a much smaller change in the protein fluorescence than the transition-state analogues. The kinetics of purine riboside binding revealed that the entire intensity drop occurs during the mixing time (4 s). This is the expected result for diffusion-controlled binding of purine riboside to adenosine deaminase (Frieden et al., 1980). In addition to a rapid intensity drop upon mixing, the binding kinetics for the transition-state inhibitors showed a slow decrease in protein fluorescence. Such a biphasic fluorescence change suggests a conformational alteration in the protein.

We also used small-molecule fluorescence quenchers to detect protein structural differences in the enzyme–inhibitor complexes. The quenching parameters (K_{sv} and f_a) for all the enzyme–inhibitor complexes were different from those for the enzyme alone, suggesting that the conformation of adenosine deaminase changes upon inhibitor binding. Iodide and trichloroethanol appeared to be more effective than acrylamide in discriminating changes in the various enzyme–inhibitor complexes. For these two quenchers, the complexes with transition-state inhibitors showed different quenching patterns from the complexes with the ground-state inhibitors. The data are consistent with the interpretation that the transition-state inhibitors cause similar protein conformational changes. For acrylamide, on the other hand, no appreciable differences were found in the quenching parameters of the enzyme–inhibitor complexes. Our acrylamide quenching data for the calf enzyme agreed qualitatively with the results of Kurz et al. (1985). However, there were quantitative differences in the Stern–Volmer constants, K_{sv} (see Table I; Kurz et al., 1985), which could be due to instrumental factors. On the basis of their K_{sv} values for calf adenosine deaminase, Kurz et al. (1985) concluded that protein conformational changes occurred upon binding of transition-state but not ground-state inhibitors. We see evidence in both the calf and human enzymes for conformational changes in the two types of enzyme–inhibitor complexes.

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Modification of Myosin Subfragment 1 Tryptophans by Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Bromide†

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Received August 12, 1986; Revised Manuscript Received December 15, 1986

ABSTRACT: Modification of tryptophanyl residues (Trps) of myosin subfragment 1 (S-1) was performed with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS). Under controlled conditions, pH 6 at 0 °C and 10-min reaction with 10–100-fold molar excess, K^+(EDTA) activity was reduced down to less than half, whereas Ca^{2+}-ATPase activity increased and acto-S-1-ATPase was not affected. The number of modified Trps (up to 2.5) agreed well with the number of 2-hydroxy-5-nitrobenzyl moieties incorporated in S-1. The thiol groups of S-1 were not affected up to 50-fold molar excess of DHNBS, thus indicating that the modification was selective for Trps. The modification of as few as one Trp caused a blue shift of the emission spectrum, accompanied by a reduction in the fluorescence quantum yield. The accessibility of Trps to the fluorescence quencher acrylamide is drastically reduced upon modification, indicating that DHNBS-reactive Trps are more "exposed" than the DHNBS-refractive ones. DHNBS modification did not seem to affect the ATP-induced tryptophan fluorescence enhancement of S-1. The effect of DHNBS modification on the intrinsic fluorescence of S-1 indicates that the modified Trps are located in a polar environment and that they may be identical with the long-lifetime Trps of Torgerson [Torgerson, P. (1984) *Biochemistry 23*, 3002–3007]. The most reactive Trp is located in the N-terminal 27-kDa fragment of the S-1 heavy chain. It might also be inferred from the above data that the nonexposed and ATP-perturbed Trp(s) is (are) located in the 50-kDa fragment.

The energy required for the contraction process is derived from hydrolysis of ATP, which occurs at a remote ATP binding site of myosin. This energy is transferred to the actin binding site, most probably in the form of a structure distortion through the S-1[1] segment, which serves as a transducer in the process (Botts et al., 1984). Such a model requires the existence of an intersite communication system. To trace this system, one should study environment-sensitive functional groups, which have the capability to "report" on conformational changes taking place in their vicinity. Trps, having environment-sensitive absorbance and fluorescence spectra, can serve as intrinsic reporter groups and have been widely used in the study of myosin structure and function. Morita

† This research was supported by grants from the U.S.–Israel Binational Science Foundation (3242) and the Muscular Dystrophy Association.

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[1] Abbreviations: ϵ_M and $\epsilon_{1\%}$, absorption coefficients, molar and by percent; DHNBS, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTE, dithioerythritol; DTT, dithiothreitol; GdmCl, guanidinium chloride; HMM, heavy meromyosin; HNB, 2-hydroxy-5-nitrobenzyl; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; kDa, kilodaltons; P_i, inorganic phosphate; S-1, chymotryptic subfragment 1; S-2, myosin subfragment 2; $NaDodSO_4$, sodium dodecyl sulfate; TES, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; Trp, tryptophanyl residue; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/87/0426-2903$01.50/0