Kinetics of Inhibition of Calf Intestinal Adenosine Deaminase by (+)- and (−)-*erythro*-9-(2-Hydroxy-3-nonyl)adenine

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ABSTRACT: The enantiomers of *erythro*-9-(2-hydroxy-3-nonyl)adenine [(+)- and (−)-EHNA) bound to adenosine deaminase (ADA) at pH 7 with concomitant changes in the optical properties of the enzyme. The association rate constant for (+)-EHNA was 2.9×10^6 M^{-1} s^{-1} and that for (−)-EHNA was 6.4×10^6 M^{-1} s^{-1}. The dissociation of (−)-EHNA·ADA or (+)-EHNA·ADA in the presence of excess coformycin was monitored by the quenching of enzyme fluorescence as coformycin·ADA was formed. The dissociation rate constants of (+)- and (−)-EHNA·ADA were 0.0054 s^{-1} and 2.7 s^{-1}, respectively. A similar value for the dissociation rate constant (0.005 s^{-1}) for (+)-EHNA·ADA was calculated from the time course for the appearance of catalytic activity after dilution of (+)-EHNA·ADA into 100 μM adenosine. The K_i values of ADA for (+)- and (−)-EHNA were similar to the dissociation constants calculated from the ratio of the respective dissociation and association rate constants. The biphasic time-dependent inhibition of the catalytic activity of ADA by (±)-EHNA [Frieden, C., Kurz, L. C., & Gilbert, H. R. (1980) *Biochemistry 19,* 5303–5309] was confirmed. However, the catalytic activity of ADA was inhibited monophasically by (+)-EHNA. Thus, the biphasic nature of the time course for inhibition of ADA by (±)-EHNA was the result of the presence of both enantiomers of the inhibitor in this assay. These kinetic data were interpreted in terms of single-step mechanisms for binding of (+)- and (−)-EHNA.

Adenosine deaminase (EC 3.5.4.4) is a zinc enzyme that catalyzes the hydrolysis of adenosine or adenosine analogues to inosine or the respective analogues (Wilson et al., 1991). The enzyme is potently inhibited by 9-(2-hydroxyl-3-nonyl)-adenine, a semi-tight-binding inhibitor discovered by Schaeffer and Schwender (1974). The four stereisomers of 9-(2-hydroxyl-3-nonyl)adenine are competitive inhibitors of ADA[1] with respect to adenosine but differ greatly in potency (Baker et al., 1981; Bessodes et al., 1982). The most potent of these inhibitors is (+)-EHNA, which has an inhibition constant of 3.2 nM for calf intestinal ADA (Bessodes et al., 1982). Initial studies that monitored the slow onset of inhibition of the enzymatic deamination of adenosine by ADA at low concentrations of (±)-EHNA suggested that (±)-EHNA binds to ADA by a single-step mechanism (Agarwal et al., 1977). Studies at higher concentrations of (±)-EHNA revealed biphasic time courses for inhibition; the limiting first-order rate constant for inhibition is 0.6 s^{-1} (Frieden et al., 1980). In contrast, other potent inhibitors of ADA inhibit the enzyme in a time-dependent monophasic manner (Frieden et al., 1980). These results supported suggestions that (±)-EHNA binds to ADA differently than other adenosine analogues (Woo & Baker, 1982; Schaeffer & Vogel, 1965). We have reinvestigated the binding of EHNA to calf intestinal ADA. The biphasic inhibition of ADA catalysis by (±)-EHNA was confirmed (Frieden et al., 1980). Inhibition of ADA catalysis by (+)-EHNA, however, was a monophasic process that was linearly dependent on the concentration of (+)-EHNA. Thus, the biphasic time courses for inhibition of ADA by (±)-EHNA were the result of the presence of both (+)- and (−)-EHNA in this assay and not of a two-step mechanism of binding of (+)-EHNA to ADA.

MATERIALS AND METHODS

Materials. (±)-EHNA was from Wellcome Research Laboratories (Research Triangle Park, NC). (+)- and (−)-EHNA were synthesized as described previously (Bessodes et al., 1982). Coformycin was provided by Dr. L. Frick of Wellcome Research Laboratories (Research Triangle Park, NC). Calf intestinal ADA was from Boehringer Mannheim Co. (Indianapolis, IN). The enzyme was judged to be homogeneous by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had a molecular weight of 34 000.

ADA Active-Site Concentration. The active-site concentration of ADA was determined by titration of the fluorescence of the enzyme (excitation 280 nm, emission 340 nm) with coformycin as described for the titration of the enzyme with deoxycoformycin (Kurz et al., 1985).

Acquisition of Spectral Data. Spectral data were collected on a Uvikon 860 spectrophotometer (Kontron, Everret, MA). Fluorescence data were collected on a Kontron SFM 25 spectrofluorometer. Rapid reactions were recorded with a SF.17MV stopped-flow spectrofluorometer (Applied Photophysics Limited, Leatherhead, UK). The time constant for the instrument was set to one-tenth of the fastest relaxation time monitored. The entrance and exit slits were 2 mm. Data were collected with 1-mm slits in the absorbance mode. Other parameters were adjusted as described in the manual from the manufacturer. Five or more individual runs were averaged for data fitting.

Dissociation of (+)-EHNA·ADA. The dissociation of (+)-ENHA·ADA was monitored by the time-dependent increase

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[1] Abbreviations: PIPES, piperazine-*N,N'*-bis[2-ethanesulfonic acid]; ADA, adenosine deaminase isozyme 1; (+)-EHNA, *erythro*-(+)-9-(2*S*-hydroxy-3*R*-nonyl)adenine; (−)-EHNA, *erythro*-(−)-9-(2*R*-hydroxy-3*S*-nonyl)adenine; (±)-EHNA, racemic *erythro*-9-(2-hydroxy-3-nonyl)-adenine; (+)-EHNA·ADA, the complex between (+)-EHNA and ADA.

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in ADA activity. (+)-EHNA·ADA was formed by incubating 100 nM ADA with 200 nM (+)-EHNA for 5 min at room temperature. This complex was diluted 500-fold into buffer that was supplemented with 100 μM adenosine and 0.02% bovine serum albumin (w/v). Deamination of adenosine was monitored at 265 nm. The deamination of adenosine by (+)-EHNA·ADA was initially zero. As (+)-EHNA dissociated from the complex, the rate of product formation increased. This process was fitted to an exponential increase in the rate of product formation to a steady state

$$[\text{product}] = A + Bt + \frac{B}{C}\exp(-Ct) \quad (1)$$

where A was a function of the initial product concentration and the amplitude of the lag phase for product formation (B/C), B was the final steady-state velocity, and C was the first-order rate constant for dissociation of (+)-EHNA from (+)-EHNA·ADA.

The dissociation of (+)-EHNA·ADA was also monitored by the time-dependent formation of coformycin·ADA. (+)-EHNA·ADA was formed by mixing 26.6 μM ADA with 30 μM (+)-EHNA. This complex was diluted 200-fold into buffer A supplemented with 18 μM coformycin. Formation of coformycin·ADA, which occurred upon dissociation of (+)-EHNA·ADA, was monitored spectrofluorometrically at 25 °C with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. These data were fitted to a single-exponential function.

Steady-State Kinetics. ADA was assayed spectrophotometrically at 265 nm ($\Delta\epsilon_{265} = 8.2\ \text{mM}^{-1}\ \text{cm}^{-1}$) with adenosine as the substrate (Spector et al., 1983). The standard buffer (buffer A) was 50 mM PIPES at pH 7.0 and 25 °C. K_m values for adenosine were determined by analyzing the complete time course of the reaction with the integrated rate equation (Spector, 1984; Cercignani & Allegrini, 1991). The initial concentration of adenosine was 100 μM.

The K_i for (−)-EHNA was calculated from the dependence of the K_m for adenosine on the concentration of (−)-EHNA. The apparent K_m for adenosine was determined at four concentrations of (−)-EHNA (0–1 μM) using the integrated rate equation. Since (−)-EHNA was a competitive inhibitor of adenosine (Bessodes et al. 1982), a plot of the apparent K_m versus (−)-EHNA was linear. The ratio of the ordinate intercept (K_m) to the slope (K_m/K_i) from this plot was the K_i for (−)-EHNA.

The steady-state inhibition of ADA by (+)-EHNA was determined with 20 μM adenosine at seven concentrations of (+)-EHNA (0–3 nM). Since the rate of equilibration of the enzyme with (+)-EHNA was slow, it was necessary to determine the steady-state velocity approximately 200 s after initiation of the reaction with adenosine or (+)-EHNA (Agarwal et al. 1977). These data were fitted to

$$v = \frac{A}{K_{app} + [(+)\text{-EHNA}]} \quad (2)$$

to yield an apparent inhibition constant K_{app} for (+)-EHNA. Since (+)-EHNA is a competitive inhibitor with respect to adenosine (Bessodes et al., 1982), K_{app} was corrected for adenosine concentration by the factor $(1 + [S]/K_m)^{-1}$ to yield the K_i for (+)-EHNA.

Time-Dependent Inhibition of Catalysis by (+)-EHNA. The K_m for adenosine was also determined from the effect that adenosine had on the pseudo-first order rate constant (k_{obs}) for the time-dependent inhibition of catalysis by (+)-EHNA. These data (five concentrations of adenosine) were fitted to

$$k_{obs} = \frac{A}{K_m + [\text{adenosine}]} \quad (3)$$

where K_m was the concentration of adenosine that produced half the maximal effect. The value of A in eq 3 was K_m times the value for k_{obs} in the absence of adenosine.

Data Analysis. Single- and double-exponential time courses (400 data points) were fitted with the software provided with the SF.17MV stopped-flow spectrofluorometer (Applied Photophysics Limited, Leatherhead, UK). The constants defined by eqs 1 and 3 were estimated by an iterative nonlinear least-squares fitting routine with equal weighting of the data (Bevington, 1969).

RESULTS

Spectral and Fluorescence Changes Associated with Binding of EHNA to ADA. When adenosine analogues bind to ADA, the optical spectra of the enzyme and/or ligand are perturbed. For example, the ultraviolet difference spectrum for binding of deoxycoformycin to ADA has an absorbance maximum at 305 nm (Frick et al., 1986). Recent investigations have used spectral changes such as these to monitor the kinetics for binding of some adenosine analogues to ADA (Kurz & Frieden, 1983; Kurz et al., 1992). When (+)- or (−)-EHNA was added to ADA, there were similar spectral changes. The ultraviolet difference spectra had absorbance maxima at approximately 280 nm ($\Delta\epsilon_{280} = 6\ \text{mM}^{-1}\ \text{cm}^{-1}$). These spectral changes were significantly larger than those changes associated with protonation of (±)-EHNA or with transfer of (±)-EHNA from a polar to a nonpolar environment (Figure 1A).

ADA fluorescence was quenched 2% upon binding of (+)-EHNA, whereas the protein fluorescence was enhanced 5% upon binding of (−)-EHNA (Figure 1B). Philips et al. (1987, 1989) previously demonstrated that (±)-EHNA quenched the fluorescence of ADA by less than 2%. In contrast to these small fluorescence changes, coformycin markedly quenched the fluorescence of the enzyme (Figure 1B).

Association Rate Constants for (+)- and (−)-EHNA. Binding of (+)-EHNA to ADA was monitored by the absorbance change at 280 nm (Figure 2A). Since the pseudo-first-order rate constant for the time-dependent binding of (+)-EHNA was linearly dependent on the concentration of (+)-EHNA (Figure 2B), the reaction was described by a bimolecular rate constant that had a value of $(2.9 \pm 0.1) \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$. Similarly, the pseudo-first-order rate constant for binding of (−)-EHNA to ADA was linearly dependent on the concentration of (−)-EHNA (Figure 2B). The association rate constant for (−)-EHNA was $(6.4 \pm 0.2) \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$.

Dissociation Rate Constant of (+)-EHNA. The dissociation rate constant for (+)-EHNA·ADA is sufficiently small ($t_{1/2} \sim 100$ s) that the approach to the steady-state rate for adenosine deamination in the presence of low concentrations of EHNA can be monitored with a conventional spectrophotometer at 265 nm (Agarwal et al., 1977). The dissociation constant for (+)-EHNA·ADA under the experimental conditions used herein was determined by this method. When (+)-EHNA·ADA was diluted into buffer A with 100 μM adenosine to a final concentration of 0.4 nM,[2] there was a time-dependent increase in catalytic activity (data not shown). These data were fitted to eq 1 with a first-order rate constant

[2] Bovine serum albumin (0.02% w/v) was added to prevent inactivation of ADA at these low protein concentrations.

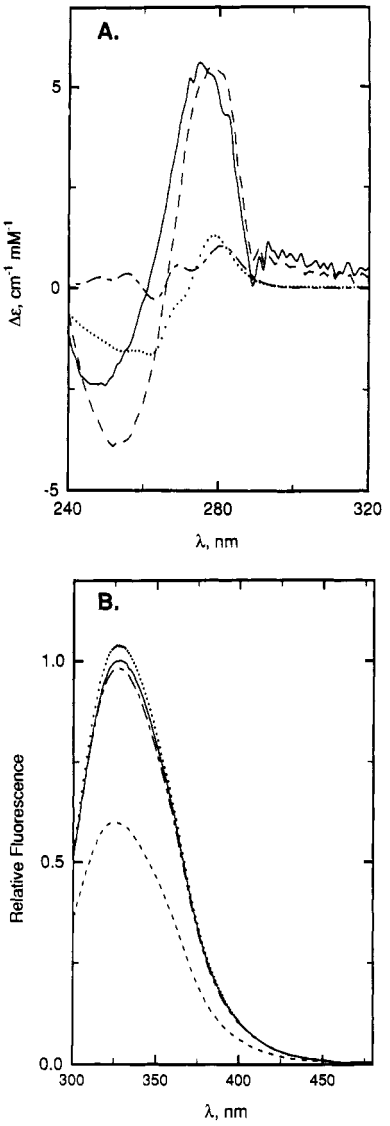

FIGURE 1: Effects of (+)- and (–)-EHNA on the optical spectra of ADA. (A) Optical absorbance spectra of 5 μM (+)-EHNA, 5 μM (–)-EHNA, 20 μM ADA, a mixture of 5 μM (+)-EHNA and 20 μM ADA, and a mixture of 5 μM (–)-EHNA and 20 μM ADA were recorded. The difference spectrum between (+)-EHNA·ADA and free (+)-EHNA and free ADA (– – –) was calculated by digitally subtracting the spectra of (+)-EHNA and ADA from the spectrum for the mixture of (+)-EHNA with ADA. The difference spectrum for (–)-EHNA·ADA (—) was calculated by a similar procedure. Extinction coefficients were calculated for bound EHNA. The difference spectrum between (+)-EHNA at pH 1 and pH 7 (– – –) as well as the difference spectrum between (+)-EHNA in 2-propanol and (+)-EHNA in buffer A (· · ·) were included for comparison. (B) Fluorescence emission spectrum of 0.9 μM ADA (excitation wavelength was 280 nm) was recorded in buffer A (—) and after addition of 2 μM (+)-EHNA (– – –), 2 μM (–)-EHNA (· · ·), or 2 μM coformycin (- - -).

of $(5 \pm 1) \times 10^{-3}$ s^{-1} for the approach to the steady state. Since the steady-state rates for adenosine deamination with untreated ADA and with EHNA-treated ADA were similar, (+)-EHNA was completely dissociated from the enzyme under these conditions. Thus, the first-order rate constant for the approach to the steady state was equal to the dissociation rate constant. The dissociation rate constant calculated from these data was similar to the value of 4.6×10^{-3} s^{-1} reported previously (Agarwal et al., 1977).

An alternative approach for determining the dissociation rate constant for (+)-EHNA was to trap ADA with coformycin as (+)-EHNA dissociated from (+)-EHNA·ADA. This

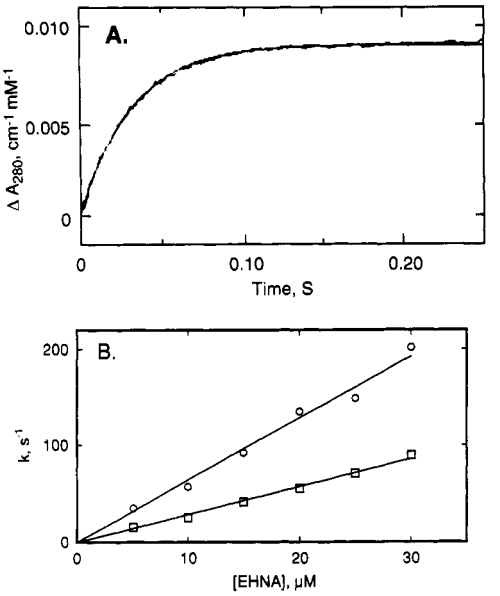

FIGURE 2: Kinetics for binding of (+)- and (–)-EHNA to ADA. (A) Time course for binding of 1.8 μM ADA and 10 μM (+)-EHNA. The reaction was monitored on the stopped-flow spectrophotometer by the absorbance change at 280 nm. These data were fitted to a single-exponential function with a first-order rate constant of 30.1 ± 0.1 s^{-1}. (B) Dependence of the pseudo-first-order rate constant for binding of (+)-EHNA (□) or (–)-EHNA (○) to ADA. The bimolecular rate constants calculated from the slope of these plots was $(2.9 \pm 0.1) \times 10^6$ M^{-1} s^{-1} for (+)-EHNA and $(6.4 \pm 0.2) \times 10^6$ M^{-1} s^{-1} for (–)-EHNA.

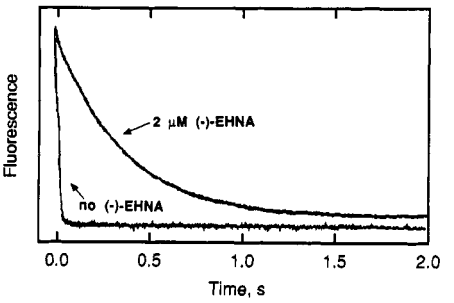

FIGURE 3: Kinetics for dissociation of (–)-EHNA·ADA. (–)-EHNA·ADA was formed by addition of 4 μM (–)-EHNA to 0.5 μM ADA. This mixture was diluted in the stopped-flow spectrophotometer with an equal volume of buffer A that had been supplemented with 200 μM coformycin. Formation of coformycin·ADA, which occurred upon dissociation of (+)-EHNA·ADA, was monitored spectrofluorometrically with an excitation wavelength of 280 nm. The data were fitted to a first-order process with a rate constant of 2.7 ● 0.1 s^{-1}. Free ADA reacted with 200 μM coformycin with a pseudo-first-order rate constant of 84 ± 1 s^{-1}.

reaction was conveniently monitored by the large fluorescence difference between (+)-EHNA·ADA and coformycin·ADA (Figure 1B). Thus, addition of 18 μM coformycin to 0.13 μM (+)-ENHA·ADA caused a time-dependent decrease in fluorescence that was described by a first-order rate constant with a value of $(5.4 \pm 0.1) \times 10^{-3}$ s^{-1}. Since the value of this apparent rate constant was not affected by decreasing the concentration of coformycin to 8 μM, it was the dissociation rate constant for (+)-EHNA.

Dissociation Rate Constant of (–)-EHNA. The dissociation rate constant for (–)-EHNA·ADA was determined by trapping free ADA with coformycin in a reaction analogous to that described above for (+)-EHNA·ADA. (–)-EHNA·ADA was formed by addition of 4 μM (–)-EHNA to 0.5 μM ADA and then mixed with an equal volume of 200 μM coformycin in the stopped-flow spectrophotometer. The reaction was monitored by the quenching of ADA fluorescence that occurred

Table I: Summary of Values for Association Rate Constants, Dissociation Rate Constants, Inhibition Constants, and Calculated Dissociation Constants for Binding of (+)-EHNA and (−)-EHNA to ADA[a]

	k_1 (10^6 M^{-1} s^{-1})	k_{-1} (s^{-1})	K_i (nM)	K_d (nM)
(+)-EHNA	2.9 ± 0.1	(5.4 ± 0.1) × 10^{-3}	1.2 ± 0.1	1.9 ± 0.1
(−)-EHNA	6.4 ± 0.2	2.7 ± 0.1	400 ± 90	420 ± 20

[a] Inhibition constant was the experimentally determined K_i. The dissociation constant was calculated from k_1 and k_{-1} by the relationship $K_d = k_{-1}/k_1$.

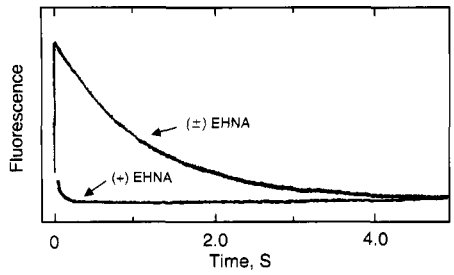

FIGURE 4: Time course for binding of (+)- or (±)-EHNA to ADA. The fluorescence changes (excitation wavelength of 280 nm) were followed on the stopped-flow spectrophotometer after 4 μM ADA was mixed with an equal volume of 40 μM (+)- or (±)-EHNA. The binding of (+)-EHNA to ADA quenched the fluorescence of ADA slightly (1.7%) in a monophasic reaction. The time-course for binding of (±)-EHNA to ADA was biphasic. The fluorescence was enhanced (3.1%) in the early phase of the reaction and was quenched in the late phase of the reaction. The first-order rate constant for the late phase of the reaction was 0.82 ± 0.01 s^{-1}.

upon formation of coformycin·ADA (Figure 3). The first-order rate constant was 2.7 s^{-1}. Because the value of this rate constant was similar when 100 μM coformycin was used to trap ADA and the fluorescence of ADA was quenched monophasically by 100 μM coformycin in the absence of (−)-EHNA with a first-order rate constant of 84 s^{-1}, the dissociation rate constant of (−)-EHNA·ADA was 2.7 s^{-1}. The dissociation constant (K_d) for (−)-EHNA and ADA was calculated to be 0.42 ± 0.02 μM from the ratio of this dissociation rate constant to the association rate constant [(6.4 ± 0.2) × 10^6 M^{-1} s^{-1}].

Inhibition Constants for (−)- and (+)-EHNA. The steady-state kinetics for inhibition of ADA by (+)-EHNA gave a K_i for (+)-EHNA of 1.2 ± 0.1 nM. The dissociation constant (K_d) for (+)-EHNA, which was calculated from the ratio of its dissociation rate constant [(5.4 ± 0.1) × 10^{-3} s^{-1}] to its association rate constant [(2.9 ± 0.1) × 10^6 M^{-1} s^{-1}] had a value of 1.9 ± 0.1 nM. The steady-state kinetics for inhibition of ADA by (−)-EHNA gave a K_i for (−)-EHNA of 400 ± 90 nM. The dissociation constant (K_d) for (−)-EHNA, which was calculated from the ratio of its dissociation rate constant (2.7 ± 0.1 s^{-1}) to its association rate constant [(6.4 ± 0.2) × 10^6 M^{-1} s^{-1}] had a value of 420 ± 20 nM. The K_i and K_d values for each inhibitor are in agreement (Table I).

Fluorescence Changes upon Binding of (+)- and (±)-EHNA to ADA. The time course for the fluorescence changes associated with binding of (±)-EHNA to ADA were biphasic (Figure 4). (±)-EHNA rapidly enhanced the fluorescence of ADA and then slowly quenched the fluorescence of the enzyme with a rate constant of 0.82 ± 0.02 s^{-1} (Figure 4). In contrast, (+)-EHNA quenched the fluorescence of ADA in a monophasic reaction (Figure 4). Since the association rate constant of ADA for (−)-EHNA was 2.2-fold larger than the association rate constant for (+)-EHNA (Figure 2B), (−)-EHNA·ADA (rapid increase in fluorescence) was preferentially formed after

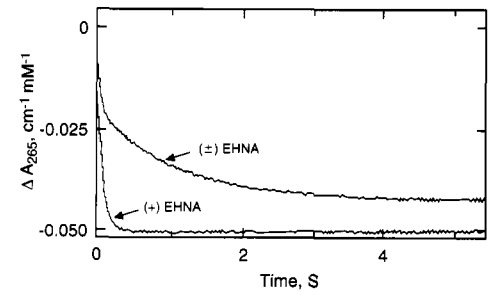

FIGURE 5: Inhibition of the ADA-catalyzed deamination of adenosine by (+)- and (±)-EHNA. ADA (2.4 μM) was mixed with an equal volume of 50 μM adenosine that contained either 20 μM (+)-EHNA or 40 μM (±)-EHNA. The deamination of adenosine was monitored by the absorbance decrease at 265 nm. In the absence of inhibitor the total absorbance decrease at 265 nm was 0.2. The time course for inhibition of the reaction by (+)-EHNA was monophasic with a first-order rate constant of 14.0 ± 0.1 s^{-1}. The time course for inhibition of adenosine deamination in the presence of (±)-EHNA was biphasic. The early phase of the reaction was characterized by a rate constant of 16.4 ± 0.3 s^{-1}, whereas the late phase of the reaction was characterized by a first-order rate constant of 0.83 ± 0.01 s^{-1}.

ADA was mixed with (±)-EHNA. Because the dissociation rate constant for (−)-EHNA·ADA was 2.7 s^{-1} and that for (+)-EHNA·ADA was only 5.4 × 10^{-3} s^{-1} (Scheme I), (+)-EHNA·ADA was the predominant species at equilibrium.

Scheme I

$$\text{(−)-EHNA + ADA} \underset{6.4 \times 10^6\ M^{-1}\ s^{-1}}{\overset{2.7\ s^{-1}}{\rightleftharpoons}} \text{(−)-EHNA·ADA}$$

$$\text{(+)-EHNA + ADA} \underset{2.9 \times 10^6\ M^{-1}\ s^{-1}}{\overset{0.0054\ s^{-1}}{\rightleftharpoons}} \text{(+)-EHNA·ADA}$$

Since the rate constant for dissociation of (+)-EHNA·ADA was much smaller that the other rate constants in Scheme I, the apparent rate constant for formation of (+)-EHNA·ADA from (−)-EHNA·ADA (the late phase of the reaction shown by Figure 4) is related to the association (k_1) and dissociation (k_2) rate constants for (−)-EHNA and the association rate constant (k_3) for (+)-EHNA by

$$k_{off} \cong \frac{k_2 k_3[\text{(+)-EHNA}]}{(k_1[\text{(−)-EHNA}] + k_2 + k_3[\text{(−)-EHNA}])} \quad (4)$$

Thus, the apparent rate constant for formation of (+)-EHNA·ADA from (−)-EHNA·ADA (k_{off}) in the presence of 40 μM (±)-EHNA was calculated from the data of Scheme I to be 0.83 s^{-1}. The calculated value was similar to the measured value of 0.82 ± 0.01 s^{-1} (Figure 4).

Kinetics of Inhibition of ADA by (+)- and (±)-EHNA. The kinetics of binding of (+)- and (±)-EHNA to ADA were determined by monitoring the time-dependent inhibition of ADA-catalyzed deamination of adenosine. This method is similar to that used by Frieden et al. (1980) and Agarwal et al. (1977). The time course for inhibition of ADA by (+)-EHNA in the presence of 25 μM adenosine was first-order process (Figure 5). The first-order rate constant for inhibition increased linearly with increasing concentration of (+)-EHNA (data not shown). The slope of a linear plot of these data was (9.5 ± 0.1) × 10^5 M^{-1} s^{-1}, which corresponded to a bimolecular rate constant of 1.9 × 10^6 M^{-1} s^{-1} after correction for the presence of adenosine (K_m = 27.7 ± 0.7 μM) in the reaction mixture. This value for the association rate constant was similar to the value of (2.9 ± 0.1) × 10^6 M^{-1} s^{-1} determined

by monitoring the binding of (+)-EHNA directly (Figure 2). The initial rate of adenosine deamination was not decreased by (+)-EHNA in these experiments. The rate constant for inhibition at a fixed concentration of (+)-EHNA decreased hyperbolically with increasing concentrations of adenosine; the K_i for adenosine calculated from these data (eq 3) was 30 $\pm$ 10 μM. The K_i for adenosine as an inhibitor of (+)-EHNA binding was similar to the K_m for adenosine as a substrate. These data were consistent with a single-step mechanism for binding of (+)-EHNA to ADA with adenosine and (+)-EHNA competing for the same site.

In contrast to the results with (+)-EHNA, the time course for inhibition of adenosine deaminase-catalyzed deamination of adenosine by (±)-EHNA was biphasic (Figure 5). The pseudo-first-order rate constant for the late phase of this reaction had a limiting value of 0.8 s^{-1} for concentrations of (±)-EHNA greater than 1 μM. These results were similar to those described by Frieden et al. (1980). The limiting value of the apparent first-order rate constant for inhibition by high concentrations of (±)-EHNA was similar to the apparent first-order rate constant for formation of (+)-EHNA·ADA from (–)-EHNA·ADA that was determined from fluorescence quenching data (Figure 4). Consequently, the apparent biphasic onset of inhibition by (±)-EHNA was a composite of the individual monophasic binding of (+)- and (–)-EHNA.

DISCUSSION

The rate constants for binding of (+)- and (–)-EHNA to ADA are summarized in Table I. The association rate constants for (+)- and (–)-EHNA were slightly less than those recently reported for 2-aminopurine riboside (8×10^6 M^{-1} s^{-1}) and adenosine (11×10^6 M^{-1} s^{-1}) (Kurz et al., 1992). The differences in the binding affinity of (+)- and (–)-EHNA were the result of their differences in their respective dissociation constants. The dissociation constants for these inhibitors calculated from the ratio of their dissociation and association rate constants were similar to the measured K_i values (Table I). Even though the kinetic data presented herein for binding of (+)- and (–)-EHNA to ADA were adequately described by a single-step mechanism, these data did not eliminate the possibility of a two-step mechanism. For example, a two-step mechanism in which the rate constant for the isomerization step was much greater than the pseudo-first-order rate constant for initial addition of inhibitor would force the kinetics to appear monophasic (Kurz et al., 1992).

Early kinetic studies on the inhibition of ADA by (±)-EHNA at low concentrations of inhibitor were consistent with a one-step mechanism for binding (Agarwal et al., 1977). However, the time courses for inhibition of ADA at higher concentrations of (±)-EHNA were biphasic (Frieden et al., 1980). These results suggested that an initial complex between (±)-EHNA and ADA is formed with a dissociation constant of 0.2 μM. The limiting value for the first-order rate constant describing the late phase of the reaction is 0.6 s^{-1} (Frieden et al., 1980). Consequently, a two-step mechanism was proposed for binding of (±)-EHNA to ADA.

The time-dependent inhibition of the catalytic activity of ADA by (+)-EHNA reported herein was a monophasic process. The pseudo-first-order rate constant for inhibition was linearly dependent on (+)-EHNA concentration. In contrast, inhibition of ADA activity by (±)-EHNA was a biphasic process. The rate constant for the late phase of this process had a limiting value of 0.8 s^{-1}, which was similar to the previously reported value of 0.6 s^{-1} (Frieden et al., 1980). The difference between the kinetics of inhibition of ADA by (+)- and (±)-EHNA was probably related to the association rate constants for (–)- and (+)-EHNA. The association rate constant of (–)-EHNA for ADA was 2.2-fold greater than that for (+)-EHNA. Consequently, (–)-EHNA·ADA accumulated transiently after (±)-EHNA was mixed with ADA. The transient increase in fluorescence of ADA with (±)-EHNA (Figure 4) was consistent with this interpretation. Subsequently, (–)-EHNA·ADA dissociated to generate catalytically active ADA. The late phase of the inhibition reaction corresponded to the trapping of ADA as (+)-EHNA·ADA. During this phase, there was significant catalytic activity due to the ADA generated by dissociation of (–)-EHNA·ADA. The free ADA was partitioned among turnover, formation of (+)-EHNA, and formation of (–)-EHNA.

In summary, (+)- and (–)-EHNA bound to ADA by a single-step mechanism. The association rate constant for (–)-EHNA was 2.2-fold larger than that for (+)-EHNA. The relative affinities of (+)- and (–)-EHNA for ADA were determined by the 500-fold difference in their respective dissociation rate constants. The apparent biphasic nature of inhibition of ADA by (±)-EHNA was the result of a composite of the individual monophasic binding of (+)- and (–)-EHNA to ADA (Scheme I).

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