Spontaneous Epimerization of (*S*)-Deoxycoformycin and Interaction of (*R*)-Deoxycoformycin, (*S*)-Deoxycoformycin, and 8-Ketodeoxycoformycin with Adenosine Deaminase†

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ABSTRACT: (*R*)-Deoxycoformycin (pentostatin), (*S*)-deoxycoformycin, and 8-ketodeoxycoformycin were compared as inhibitors of calf intestine adenosine deaminase. In contrast to (*R*)-deoxycoformycin, which had been demonstrated as a tight-binding inhibitor with a dissociation constant of 2.5×10^{-12} M [Agarwal, R. P., Spector, T., & Parks, R. E., Jr. (1977) *Biochem. Pharmacol. 26*, 359–367], (*S*)-deoxycoformycin and 8-ketodeoxycoformycin are slope-linear competitive inhibitors with respect to adenosine. The kinetic constants are 33 μM for inhibition by (*S*)-deoxycoformycin, 43 μM for 8-ketodeoxycoformycin, and 16 μM for the K_m for adenosine. The stereochemistry of carbon 8 of the diazepine ring therefore causes a (1.3×10^7)-fold change in the affinity for the enzyme which is specific for the *R* configuration. This difference is attributed to an induced conformational change which cannot be initiated by the *S* isomer or the 8-keto analogue of (*R*)-deoxycoformycin. The studies were complicated by the need to remove traces of tight-binding inhibitor(s) from (*S*)-deoxycoformycin, since as little as 0.001% of the *R* isomer causes significant inhibition. The *R* and *S* isomers of deoxycoformycin are unstable in neutral or mildly acidic aqueous solutions. Isomerization of the secondary hydroxyl at carbon 8 of the diazepine ring is one of the reactions, resulting in *S* to *R* and *R* to *S* conversions for deoxycoformycins. Opening of the aglycon is also a major reaction. The tight-binding inhibitor generated from (*S*)-deoxycoformycin was identified as (*R*)-deoxycoformycin by high-pressure liquid chromatography, spectroscopy, circular dichroism, and chemical criteria. The enzyme did not catalyze epimerization of (*S*)- or (*R*)-deoxycoformycins. Dialysis of (*S*)-deoxycoformycin against excess enzyme provided an efficient method for removing (*R*)-deoxycoformycin or other tight-binding inhibitors. This technique provides a general solution for removing traces of tight-binding inhibitors from larger quantities of weaker inhibitors.

The chemical synthesis of (*R*)-deoxycoformycin [pentostatin, (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol] was first reported by Baker & Putt (1979). The procedure resulted in a pair of stereoisomers at the 8-position of the diazepine ring (Figure 1) which were separated in the final steps of the preparation. The *R* isomer was identical in all respects with the authentic antibiotic originally isolated from culture filtrates of *Streptomcyes antibioticus* NRRL 3238 (Woo et al., 1974). Chemically synthesized (*R*)-deoxycoformycin demonstrated the same inhibitory property toward adenosine deaminase as the natural compound, consistent with the stereochemical assignment originally made by X-ray diffraction (Woo et al., 1974; Dion et al., 1977). Agarwal et al. (1977) and Frieden et al. (1980) have demonstrated that (*R*)-deoxycoformycin is a tight-binding inhibitor of adenosine deaminase, giving an equilibrium dissociation constant of 2.5×10^{-12} M. The formation of the initial complex of enzyme and inhibitor is thought to be rapidly reversible with a dissociation constant of greater than 100 μM followed by a slow transition which is accompanied by the tight-binding of the inhibitor (Frieden et al., 1980).

Inhibition of adenosine deaminase by (*S*)-deoxycoformycin has not been quantitated; however, Chan et al. (1982) reported that chromatographically pure *S* isomer was less than 0.1% as potent as the *R* isomer in causing tight-binding inhibition of adenosine deaminase. These authors also were aware of the potential problem of a minor contamination of (*S*)-deoxycoformycin with the *R* isomer. It was of interest to quantitate the interaction of the *R* and *S* isomers of deoxycoformycin since the inhibition constants provide information on the difference between ground-state and transition-state structures of enzyme–inhibitor complexes where the only difference in inhibitor structure is the configuration of the carbon normally involved in the active site chemistry. The results reported here provide methods for removing traces of a tight-binding inhibitor from a large excess of a more modest inhibitor and permit quantitation of the kinetic constants for both. In the case of (*R*)- and (*S*)-deoxycoformycin, the *R*-isomer is a tight-binding inhibitor while the *S* isomer and the 8-keto analogue inhibit with constants similar to the K_m for substrate and are competitive inhibitors with respect to adenosine. The results also demonstrate that the *R* and *S* isomers of deoxycoformycin isomerize under conditions normally used for assay and storage of the compounds. These findings should be useful in understanding the metabolic fates of (*R*)-deoxycoformycin which has recently been involved in clinical trials (Cancer Treatment Symposia, 1984).

Materials and Methods

(R)- and (S)-Deoxycoformycin and 8-Ketodeoxycoformycin. (*R*)-Deoxycoformycin was the natural product

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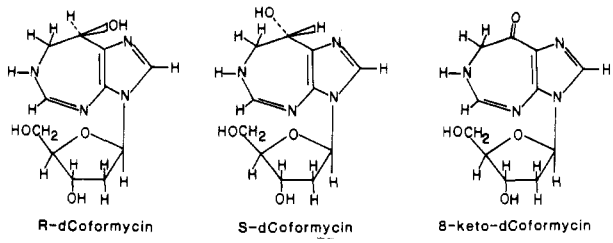

FIGURE 1: Structures of (*R*)- and (*S*)-deoxycoformycin and 8-keto-deoxycoformycin. The asymmetric carbon that determines the *R* or *S* configuration is C8 of the molecule.

isolated from *S. antibioticus* and provided by the Warner-Lambert/Parke-Davis Co. (*S*)-Deoxycoformycin and 8-keto-deoxycoformycin were synthesized by the chemical procedure of Baker & co-workers (Chan et al., 1982). The *R* and *S* diastereomers were separated by using preparative chromatography on reverse-phase octadecylsilyl-derivitized silica gel and 92.5:7.5 H_2O–methanol, pH 7.5, as solvent (Chan et al., 1982). Analytical separation of the *R* and *S* isomers was also accomplished by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column (0.25 × 40 cm) using an eluant of 2.5:97.5 CH_3CN–5 mM potassium phosphate, pH 7.0. By use of this system the retention time of the *R* and *S* isomers differs by approximately 6 min (see Figure 4). The concentrations of (*R*)- and (*S*)-deoxycoformycins were determined by using a molar extinction coefficient of 8200 at 282 nm and a pH of 7.5, and the concentration of 8-keto-deoxycoformycin was determined in methanol by using a molar extinction coefficient of 23 100 at 232 nm (Chan et al., 1982).

Enzyme. Adenosine deaminase from calf intestine was purchased from Sigma Chemical Co. and dialyzed against 20 or 40 mM potassium phosphate, pH 7.0, for 24 to 72 h prior to use. The concentration of enzyme binding sites was determined by titration with (*R*)-deoxycoformycin as described below. The quantity of enzyme protein was estimated from the absorbance at 280 nm by using the value of 0.916 absorbance unit for a 1 mg/mL solution of the enzyme (Wolfenden et al., 1977). The molecular weight of the enzyme was assumed to be 35 000 (Zielke & Suelter, 1971). Active site titration with (*R*)-deoxycoformycin (see below) indicted that the enzyme preparation was 49% active enzyme. Similar studies by Frieden et al. (1980) have established that inactive protein in this preparation does not bind the inhibitor.

Enzyme Assay. Adenosine deaminase activity was measured in assay mixtures containing 20 mM potassium phosphate, pH 7.0, 100 μM adenosine, and 1 μM EDTA. Reaction mixtures of 0.3–1 mL in microcuvettes were warmed to 30 °C before the reaction was initiated by the addition of from 0.5 to 10 μL of enzyme which was freshly diluted into the potassium phosphate buffer or buffer containing 0.5 mg/mL bovine serum albumin. Reaction rates were determined from the rate of absorbance change at 267 nm. Under the conditions of the assay, conversion of 1 mM adenosine to 1 mM inosine resulted in a decrease of 6.5 absorbance units/cm.

Purification of (S)-Deoxycoformycin with Adenosine Deaminase. Adenosine deaminase which had been dialyzed against 40 mM potassium phosphate, pH 7.0, was added to (*S*)-deoxycoformycin in 20–30 mM potassium phosphate, pH 7.0. The solutions were stored on ice for 30 min and were added to an ultrafiltration apparatus (Schramm, 1976) which had been cooled on ice and which contained a Spectapor 2 dialysis membrane rated at M_r 12 000–14 000 porosity. In some experiments, adenosine deaminase and (*S*)-deoxycoformycin were treated as described above, except all operations were at room temperature. In a typical experiment the mixture prepared for ultrafiltration contained 18 mM (*S*)-deoxycoformycin, 28 mM potassium phosphate, pH 7.0, and 4.1 μM adenosine deaminase (as determined by active site titration) in a total volume of 89 μL. The solution was forced through the membrane by 20 psi of N_2 pressure, and the filtrate was collected. The filtration time was approximately 1 h. The filtrate contained 19 mM (S)-deoxycoformycin (due to H_2O evaporation) as judged by the ultraviolet absorbance spectrum, and no enzyme activity could be detected in the filtrate.

Tight-Binding Inhibition by Deoxycoformycin. Reaction mixtures for inactivation experiments contained dialyzed adenosine deaminase at the desired concentration, 20 mM potassium phosphate, pH 7.0, and the desired concentration of (*R*)-deoxycoformycin or (*S*)-deoxycoformycin in a volume of 20 μL. The mixtures were incubated on ice, and samples of 1 μL were removed at timed intervals and assayed in 0.3 mL of reaction mixture which contained 140 μM adenosine in 20 mM potassium phosphate, pH 7.0. Samples were taken from each incubation mixture at intervals from 12 to 112 min to establish that near equilibrium had been reached between the enzyme and inhibitor. The reaction was considered to be at equilibrium when the enzyme activity remained constant as a function of time. Three initial rates were averaged to give the activities with the standard errors shown in the figures. Controls were run to establish that the enzyme activity was stable when incubated for the same time in the absence of inhibitor.

Circular Dichroism. The stereochemical assignment of *R* and *S* isomers of deoxycoformycin was made by circular dichroism. Compounds were collected following their resolution by HPLC. The samples were lyophilized and dissolved in H_2O or D_2O. Samples of 0.8 mL were placed in a 1-cm quartz cuvette and analyzed in a Jasco J-40 circular dichroism spectropolarimeter.

Analysis of Deoxyribose. Deoxyribose was determined by a modification of the diphenylamine method summarized by Blakely (1966). Samples contained 10–20 nmol of the unknown compound in a final volume of 1.5 mL. Samples were incubated for 37 °C for 4 h or overnight before measuring the absorbance at 595 nm. Deoxyadenosine monophosphate was used as a standard for the assay.

RESULTS

Tight-Binding Inhibition of Adenosine Deaminase. Addition of 0.1 μM (*R*)-deoxycoformycin or (*S*)-deoxycoformycin to reaction mixtures that contained adenosine deaminase (10^{-10} or 5×10^{-9} M) and adenosine (100 μM) as substrate resulted in the previously reported time-dependent inhibition by the *R* isomer [e.g., see Cha et al. (1975)] but no significant inhibition by an equal concentration of the *S*-isomer. Increasing concentrations of the *S* isomer to 10 μM had no detectable effect on the initial rate of the adenosine deaminase reaction. However, incubation of adenosine deaminase with increasing concentrations of the *S* isomer caused the enzyme to undergo a time-dependent loss of activity similar to that seen at much lower concentrations of (*R*)-deoxycoformycin (compare Figures 2 and 3). Incubation of adenosine deaminase with stoichiometric concentrations of (*R*)-deoxycoformycin causes tight-binding inhibition which is not reversed during subsequent dilution and assay of the enzyme. This property allows quantitation of enzyme binding site concentration as shown in Figure 2. The inactivation of enzyme by (*S*)-deoxycoformcyin could be caused either by a slow inactivation of enzyme by the *S* isomer or by a small contamination of the *S* isomer with *R* isomer. The apparent inactivation of adenosine deaminase by (*S*)-deoxycoformycin seen in Figure 3A

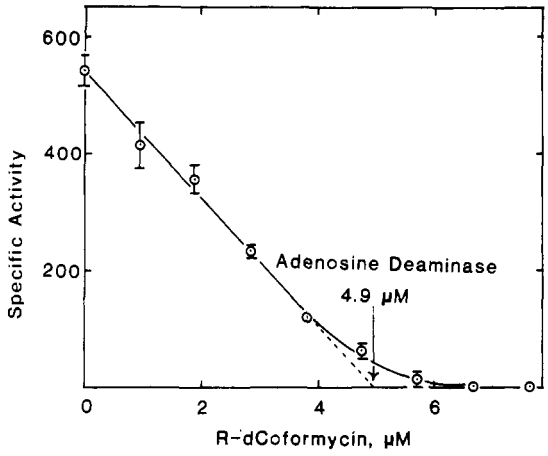

FIGURE 2: Active site titration of adenosine deaminase with (*R*)-deoxycoformycin. Dialyzed adenosine deaminase, nominal concentration 10 μM based on a molecular weight of 35 000, was incubated for approximately 2 h at 0 °C with (*R*)-deoxycoformycin and assayed as outlined under Materials and Methods. The decrease in enzymatic activity was complete in all cases by 20 min of incubation. Each point is the average of three activity determinations. The error bars represent two standard deviations from the mean. Where error bars are missing, the error falls within the graphic data point. Specific activity is expressed in micromoles of product formed per minute per milligram of active enzyme protein.

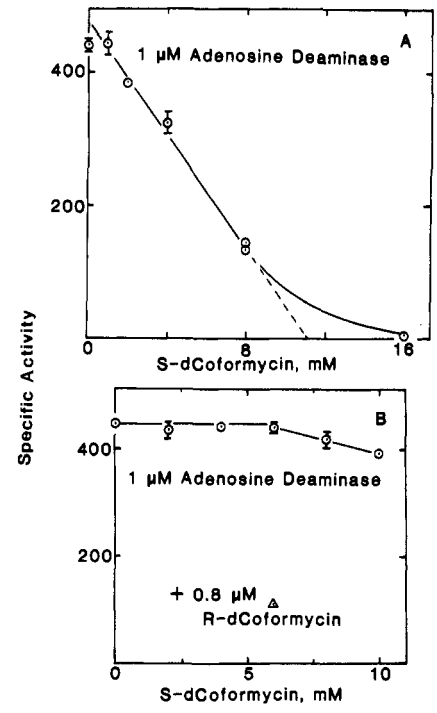

FIGURE 3: Inactivation of adenosine deaminase by incubation with (*S*)-deoxycoformycin. Dialyzed adenosine deaminase was incubated with (*S*)-deoxycoformycin and assayed for loss of enzymatic activity as described under Materials and Methods and in the legend to Figure 2. Incubation of adenosine deaminase in buffer without (*S*)-deoxycoformycin gave no loss of activity. The abscissa intercept in panel A is 11.1 mM (*S*)-deoxycoformycin. The standard error bars have the meaning as in Figure 2 except for the points at 8 mM, where duplicate determinations are recorded. The experiment in panel B (circles) was the same as in panel A except that the (*S*)-deoxycoformycin had been treated with adenosine deaminase as described under Materials Methods. The triangle labeled "+0.8 μM *R*-dCoformycin" is the result obtained by incubating adenosine deaminase with 6 mM (*S*)-deoxycoformycin *and* 0.8 μM (*R*)-deoxycoformycin.

would occur if 0.009% of the (*S*)-deoxycoformycin were (*R*)-deoxycoformycin, since the abscissa intercept of Figure 3A indicated that 11 mM (*S*)-deoxycoformycin was required to inactivate 1 μM adenosine deaminase. To distinguish these possibilities, a solution of *S* isomer (18 mM) was incubated with adenosine deaminase (4.1 μM) for 30 min to permit interaction of enzyme and inhibitor. The resulting (*S*)-deoxycoformycin was separated from the enzyme by ultrafiltration (see Materials and Methods) and tested for the ability to inhibit adenosine deaminase by measuring the extent of enzyme inactivation following incubation of adenosine deaminase and (*S*)-deoxycoformycin (Figure 3B). The treatment of *S*-deoxycoformycin with adenosine deaminase caused no detectable change in the ultraviolet absorbance spectrum of the compound and did not result in a significant change in the concentration of (*S*)-deoxycoformycin.

Incubation of (*S*)-deoxycoformycin with adenosine deaminase was effective in removing most of the inhibitor from the *S* isomer. A minor component of the solution, and not (*S*)-deoxycoformycin, was therefore responsible for the inactivation seen in Figure 3A. Addition of 0.8 μM (*R*)-deoxycoformycin to 6 mM (*S*)-deoxycoformycin demonstrated that the (*R*)-isomer causes tight-binding inhibition in the presence of a large excess of the *S* isomer (Figure 3B). These results are consistent with the observed inactivation of adenosine deaminase being due to a minor impurity which is a tight-binding inhibitor of adenosine deaminase. Incubation of enzyme with the *S* isomer (Figure 3A) demonstrated that the sample contained 90 ppm of the tight-binding inhibitor prior to purification. Following treatment with adenosine deaminase at a molar ratio of 230 parts enzyme per million parts of (*S*)-deoxycoformycin, the contaminating inhibitor was reduced from 90 to 8 ppm. Increasing the ratio of enzyme to 770 parts enzyme per million parts of the *S* isomer reduced the tight-binding inhibitor to 4.2 ppm. Treatment of the (*S*)-deoxycoformycin with adenosine deaminase was not completely effective in removing inhibitor, even though the enzyme was in relatively large excess compared to the effective dissociation constant of 2.5 pM for the complex of adenosine deaminase and (*R*)-deoxycoformycin (Frieden et al., 1980). Changing the temperature at which enzyme and inhibitor were incubated from 2 °C to room temperature had no effect on the efficiency of this process. Equilibrium calculations (see Discussion) indicated that this quantity of enzyme would reduce tight-binding inhibitors with the same affinity as (*R*)-deoxycoformycin to below detectable levels and suggested that other processes were occurring.

Formation of (R)-Deoxycoformycin from (S)-Deoxycoformycin. Samples of (*S*)-deoxycoformycin that had been stored at −20 °C or at 0 °C contained increased amounts of tight-binding inhibitor. For example, storage for 10 months at −20 °C caused an increase in tight-binding inhibitor from 0.009% to 0.2% of the (*S*)-deoxycoformycin concentration. Purified (*S*)-deoxycoformycin which contained only traces of tight-binding inhibitor was readily converted to the tight-binding inhibitor by heating at 80 °C at pH values from 3 to 8. The rate was most rapid at pH 3 and was 3 times more rapid at pH 3 than at pH 8. Approximately 6% of (*S*)-deoxycoformycin was converted to the inhibitor of adenosine deaminase when a solution (pH 3.0 in 20 mM potassium phosphate) was heated to 80 °C for 160 min. At pH 3.0, 30% of the ultraviolet absorbance was lost during this incubation, while at pH 8.0, the loss of absorbance was 22%.

The tight-binding inhibitor was characterized as (*R*)-deoxycoformycin by HPLC, circular dichroism, the ultraviolet absorbance spectrum, and analysis for deoxyribose. Samples of (*S*)-deoxycoformycin in 20 mM potassium phosphate, pH 3.0, were incubated at 80 °C for 6 h and assayed for the presence of (*R*)-deoxycoformycin by HPLC and by the ability

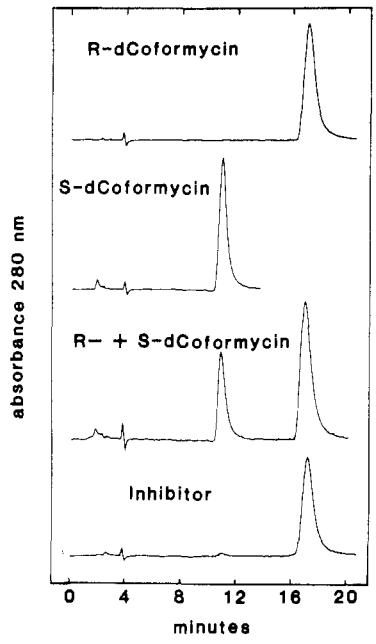

FIGURE 4: Separation of (*R*)- and (*S*)-deoxycoformycin by HPLC. Samples (20 μL of approximately 100 μM) of (*R*)-deoxycoformycin, (*S*)-deoxycoformycin, and a mixture of the isomers were applied to a Beckman Altex C-18 reverse-phase column and eluted as described under Materials and Methods. The inhibitor of adenosine deaminase generated from (*S*)-deoxycoformycin was resolved from (*S*)-deoxycoformycin by preparative HPLC, lyophilized, and rerun on analytical HPLC. The inhibitor elutes in the same position as (*R*)-deoxycoformycin (lower HPLC profile).

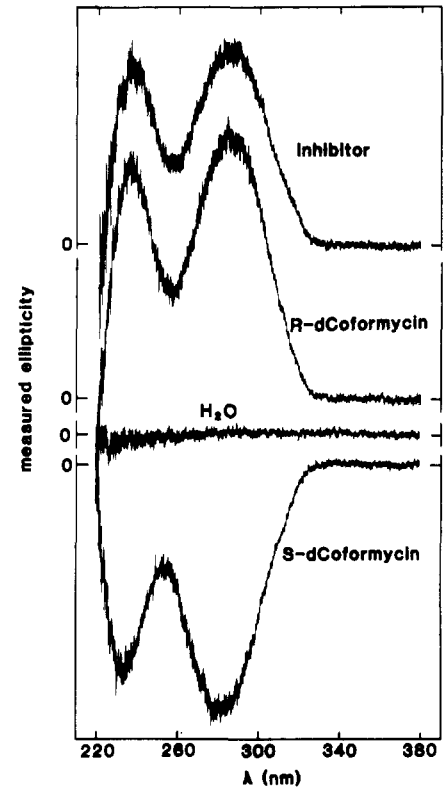

FIGURE 5: Circular dichroism of deoxycoformycins. Samples contained approximately 100 μM of the desired compound based on the absorbance at 280 nm. The sample chamber contained 0.8 mL of sample in a 1-cm path-length sample holder. Spectra were collected at room temperature with a chart speed of 1 cm/min, 10 nm/cm, sensitivity 20×10^{-1} m deg /cm, and a band width of 1 nm. All samples were recorded with the same instrument settings. The compounds were dissolved in D_2O as solvent.

to inhibit adenosine deaminase (see Figures 2 and 3). The resolution of *R* and *S* isomers of deoxycoformycin by HPLC and the coelution of the inhibitor with authentic (*R*)-deoxycoformycin are shown in Figure 4. To further establish the identity of the inhibitor, the ultraviolet and circular dichroism spectra were compared for (*S*)-deoxycoformycin (*R*)-deoxycoformycin, and the inhibitor. The ultraviolet spectra all gave maxima at 283 nm and were not significantly different (data not shown). Both (*R*)-deoxycoformycin and the inhibitor gave the same circular dichroism spectra with positive ellipticity while (*S*)-deoxycoformycin gave the same spectral absorption peaks but gave negative ellipticity (Figure 5). The inhibitor was analyzed for the presence of deoxyribose, as described under Materials and Methods, following separation from (*S*)-deoxycoformycin by HPLC. The results indicated that the deoxyribose content of the inhibitor was equimolar with that of standard (*R*)-deoxycoformycin. Control experiments with added deoxyribose demonstrated that free deoxyribose eluted at approximately 3 min by using the HPLC system shown in Figure 4.

To test whether the enzyme converts the *S* isomer to (*R*)-deoxycoformycin or other tight-binding inhibitor, 50 μM adenosine deaminase and 69 μM (*S*)-deoxycoformycin in 20 mM potassium phosphate, pH 7.0, were incubated for 5 min at 30 °C. The enzyme was denatured by treatment with 0.1 M NaOH, the sample was neutralized with HCl, and the protein was removed by ultrafiltration. None of the (*S*)-deoxycoformycin was lost during the incubation, and the (*S*)-deoxycoformycin which was isolated following ultrafiltration coeluted with the authentic compound when analyzed by high-pressure liquid chromatography. No (*R*)-deoxycoformycin or other products that absorb at 256 nm were detected. Incubation of adenosine deaminase (50 μM) with (*S*)-deoxycoformycin (69 μM) and adenosine (1 mM) likewise had no effect on the enzyme activity when samples of the incubation mixture were sampled over a 30-min period [20 min with (*S*)-deoxycoformycin and 10 additional min after the addition of adenosine]. The results indicated that adenosine deaminase does not catalyze the formation of (*R*)-deoxycoformycin or other tight-binding inhibitor from (*S*)-deoxycoformycin.

Inhibition Constant for (S)-Deoxycoformycin. The results described above (considered further under Discussion) indicated that valid initial rate studies could be done with (*S*)-deoxycoformycin which contains 10 ppm or less of *R*-deoxycoformycin, provided that the concentration of the *S* isomer does not exceed 200 μM, that the enzyme concentration is maintained below 1 nM, and that incubation times are short. Rapid measurements of initial rates were essential since the continuous formation of (*R*)-deoxycoformycin caused relatively short initial rate periods. Initial rates were maintained for approximately 1 min or 30 s when the concentrations of *S* isomer were 50 or 100 μM, respectively. (*S*)-Deoxycoformycin was maintained at 100 μM or below to enable initial rate measurements. (*S*)-Deoxycoformycin preparations with 8 or 4.2 ppm of the *R* isomer were used for the steady-state kinetic studies. The results of one such study are given in Figure 6. Analysis of the data indicated that adenosine deaminase is inhibited by (*S*)-deoxycoformycin as a competitive inhibitor with respect to adenosine to give slope-linear inhibition with an inhibition constant of 33 ± 4 μM and a K_m of 16 ± 2 μM for adenosine. The Gibbs free energy for the formation of the adenosine deaminase and (*S*)-deoxycoformycin complex can be calculated to be −6.2 kcal/mol from this inhibition constant.

Inhibition of Adenosine Deaminase by 8-Ketodeoxycoformycin. The inhibition of adenosine deaminase by 8-keto-

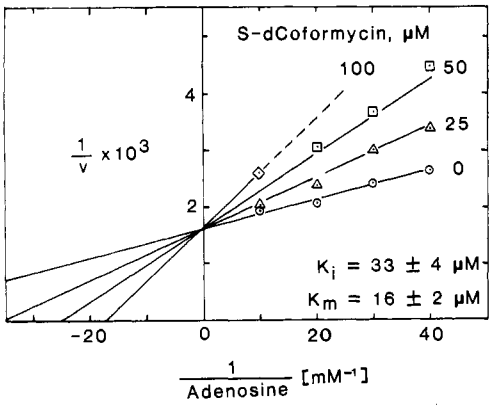

FIGURE 6: Steady-state inhibition of adenosine deaminase by (*S*)-deoxycoformycin. Initial rates were measured in duplicate from experiments as described under Materials and Methods. The initial rates were derived from the tangent to the curve during the first 30 s of reaction. The data points are the average of two determinations. The lines represent the best fit of the data to the equation for competitive inhibition using the COMP program of Cleland (1979).

deoxycoformycin was competitive with a slope-linear inhibition constant at 43 ± 5 μM. Incubation of catalytic amounts of enzyme with a large molar excess of the 8-keto analogue caused no inactivation. The addition of 100 μM 8-keto-deoxycoformycin to reaction mixtures containing 100 μM adenosine did not prevent the complete hydrolysis of adenosine by adenosine deaminase.

DISCUSSION

Quantitation of the inhibition of adenosine deaminase by the *S* isomer of deoxycoformycin was complicated by the presence of (*R*)-deoxycoformycin, a transition-state inhibitor for the enzyme. This problem was resolved by binding tight-binding inhibitor(s) to adenosine deaminase and by ultrafiltration to separate the purified (*S*)-deoxycoformycin from the enzyme. The efficiency of this method can be demonstrated by considering the equilibrium of the two inhibitors with adenosine deaminase. If inhibitor I represents (*S*)-deoxycoformycin and inhibitor T represents (*R*)-deoxycoformycin with dissociation constants of 33 μM and 2.5 pM, respectively, and K_i and K_t represent the dissociation constants, the competition can be written as

$$ET \overset{K_t}{\rightleftharpoons} T + E + I \overset{K_i}{\rightleftharpoons} EI$$

The concentration of the enzyme-bound inhibitor, ET, can be obtained from solution of the quadratic equation:

$$[ET]^2 - (E_t + T + K_t + K_t I/K_i)[ET] + E_t T = 0$$

where ET is the tightly bound complex of adenosine deaminase and (*R*)-deoxycoformycin, E_t is total enzyme concentration, T is the initial concentration of tight-binding inhibitor, K_t is the dissociation constant for the ET complex, I is the free concentration of (*R*)-deoxycoformycin, and K_i is the dissociation constant for the EI complex. Solution of this equation using the conditions of an ultrafiltration experiment, where I = 18 mM, T = 1.6 μM, and E_t = 4.1 μM, indicates that the ET complex will contain 1.599 μM (*R*)-deoxycoformycin. Experimentally, this efficiency was not observed, which suggested that the equilibrium binding scheme is an oversimplification of the interaction of deoxycoformycins with adenosine deaminase.

The reappearance of a tight-binding inhibitor in stored aqueous solutions of (*S*)-deoxycoformycin indicated that a product which is formed continuously from (*S*)-deoxycoformycin is responsible for this inhibition. The tight-binding inhibitor was demonstrated to be (*R*)-deoxycoformycin by criteria which eliminate other likely products of (*S*)-deoxycoformcyin degradation. Deoxycoformycins are known to be susceptible to solvolysis of the N-glycosidic bond and to opening of the aglycon (Dion et al., 1977; Woo et al., 1974). Inhibition by the aglycon was eliminated by the presence of deoxyribose in the compound, and inhibition by a ring-opened derivative was eliminated by the ultraviolet absorbance spectrum. The circular dichroism spectrum clearly demonstrates the isomerization from (*S*)- to (*R*)-deoxycoformycin. Dion et al. (1977) originally reported that 2% of (*R*)-deoxycoformycin is lost in 24 h in aqueous solution at room temperature at pH 6.9. The loss of (*S*)-deoxycoformycin would be expected to occur at a similar rate. The continued formation of (*R*)-deoxycoformycin from (*S*)-deoxycoformycin explains the incomplete removal of tight-binding inhibitor from (*S*)-deoxycoformycin with adenosine deaminase. This process also explains the time-dependent tight-binding inhibition of adenosine deaminase activity when assayed in the presence of 50–100 μM (*S*)-deoxycoformycin. The rate of conversion of (*S*)-deoxycoformycin to (*R*)-deoxycoformycin is approximately 4×10^{-6} min^{-1} when calculated for 30 °C (assuming a 2-fold change in reaction rate per 10 °C). At 100 μM (*S*)-deoxycoformycin, approximately 4×10^{-10} M tight-binding inhibitor will be formed per min. Since enzyme concentrations of 10^{-9}–10^{-10} M were typically used in steady-state kinetic measurements, incubation of (*S*)-deoxycoformycin for several minutes at 30 °C would generate sufficient inhibitor to cause tight-binding inhibition of a significant fraction of the enzyme at equilibrium. Frieden et al. (1980) have indicated that the rate constant for formation of tightly bound adenosine deaminase and (*R*)-deoxycoformycin is 2×10^6 mol^{-1} s^{-1}. Under conditions where adenosine deaminase and (*R*)-deoxycoformycin are both 10^{-9} M, initial rates would prevail for approximately 50 s. Thus 10^{-9} M (*R*)-deoxycoformycin can be tolerated in initial rate determinations provided that the reaction is started with enzyme. At concentrations of (*S*)-deoxycoformycin above 200 μM, the rate of formation of (*R*)-deoxycoformycin is too fast to permit normal initial rate kinetics; however, stopped-flow studies could still be done.

The possibility that the enzyme catalyzed the conversion of (*S*)-deoxycoformycin to (*R*)-deoxycoformycin was eliminated by incubating stoichiometric quantities of enzyme and (*S*)-deoxycoformycin both in the presence and in the absence of adenosine. No inactivation of enzyme occurred, and no (*R*)-deoxycoformycin or other breakdown products were detected by HPLC following such experiments. These results also indicate that contaminating enzymes (such as glycohydrolases)[1] are not a significant factor, especially in steady-state kinetic experiments. Attack of an enzyme-activated water or hydroxide ion on C8 of the diazepine ring of (*S*)-deoxycoformycin must also be considered since a proposed mechanism for adenosine deaminase involves an attack from the face of the molecule which generates (*R*)-deoxycoformycin. The possibility that an 8-dihydroxy intermediate is formed from (*S*)-deoxycoformycin which decomposes to the 8-keto

[1] A reviewer has suggested the possibility that (*R*)-deoxycoformycin which is present as a contaminant in (*S*)-deoxycoformycin might be hydrolyzed to the free base by a nonspecific glycohydrolase during dialysis against impure adenosine deaminase. The free base might then be a tight-binding inhibitor of somewhat lesser affinity than (*R*)-deoxycoformycin and thus account for the incomplete removal of tight-binding inhibitor. The presence of adenosine deaminase in excess of the concentration of (*R*)-deoxycoformycin assures that virtually none of the inhibitor is free in solution (see Discussion). Thus, the (*R*)-deoxycoformycin would be unavailable for reaction with a glycohydrolase.

analogue can be excluded from the high-pressure liquid chromatography analysis of enzyme-treated (*S*)-deoxycoformycin and from the relatively weak inhibition by 8-ketodeoxycoformycin.

Initial rate studies with (*R*)-deoxycoformycin and partially purified (*S*)-deoxycoformycin have established that, under equilibrium conditions, the *R* isomer binds 1.3×10^7 times as tightly to adenosine deaminase as does the *S* isomer. Both of these inhibitors have the same composition of chemical determinants, and the formation of an additional hydrogen (or other) bond to the epimeric hydroxyl group would be insufficient to account for this difference in binding.

Inhibition by 8-ketodeoxycoformycin (K_i = 43 μM) is similar to that with (*S*)-deoxycoformycin (K_i = 33 μM), and both compounds have inhibition constants similar to the K_m value for adenosine (K_m = 16 μM). These constants therefore represent the formation of "ground-state" complexes (Frieden et al., 1980), in which major conformational changes of protein and bound substrate or substrate analogues have not yet occurred. The large difference in binding energy of 9.9 kcal/mol between *R* and *S* isomers of deoxycoformycin must therefore involve the inhibitor-induced conformational change of enzyme which requires the *R* configuration at C8 of the inhibitor. This is an example of what Jencks (1975) has called the "Circe" effect and what is commonly called transition-state inhibition (Wolfenden, 1972). The differences in binding for (*R*)- and (*S*)-deoxycoformycin provide a unique example of the energy of interaction for ground-state and transition-state interactions with adenosine deaminase. However, the energy difference in these binding states does not necessarily represent the energy difference between the Michaelis complex and the actual transition state with adenosine, since very small differences in structure can have large effects on binding affinity.

In summary, the configuration of the 8-hydroxyl group of deoxycoformycin is crucial for the analogue-induced conformational change leading to the formation of the tightly bound complex. The free energy of these interactions is −6.2 kcal/mol for the *S* isomer and −16.1 kcal/mol for the *R* isomer for a difference of 9.9 kcal/mol. This provides an estimate of the energy which can be applied to substrate distortion in the formation of the transition-state complex with this analogue. (*S*)-Deoxycoformycin is unstable under normal assay conditions and forms (*R*)-deoxycoformycin by isomerization at carbon 8 of the diazepine ring. The tight-binding inhibitor is formed at a rate that causes inhibition of adenosine deaminase under normal assay conditions. A general method is described for removing tight-binding inhibitors from more modest inhibitors. Since (*R*)-deoxycoformycin is currently in clinical trials as an adenosine deaminase inhibitor, the ability of (*S*)-deoxycoformycin to provide tight-binding inhibition of the enzyme may also prove useful.

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Registry No. Pentostatin, 53910-25-1; (*S*)-deoxycoformycin, 69196-04-9; 8-ketodeoxycoformycin, 69196-03-8; adenosine, 58-61-7; adenosine deaminase, 9026-93-1.

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