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Dihydrofolate Reductase Hysteresis and Its Effect on Inhibitor Binding Analyses†

David P. Baccanari* and Suzanne S. Joyner

ABSTRACT: *Escherichia coli* dihydrofolate reductase was shown to follow slow transient kinetics (hysteresis). Nonlinear reaction velocities were detected during the enzyme assay and required 10–15 min to reach a steady-state rate. The degree of hysteresis was influenced by the enzyme concentration and the order of substrate addition. Incubation of the enzyme with NADPH before addition of dihydrofolate resulted in slow initial velocities that increased up to 2-fold during the course of the assay. Increasing the enzyme concentration from 0.2 to 1 nM resulted in diminished hysteresis. NADPH-initiated reactions were linear at all enzyme concentrations tested. Certain drugs had profound effects on hysteresis. Pyrimethamine practically eliminated the hysteresis of dihydrofolate-started reactions, whereas trimethoprim augmented the nonlinearities in the sense that hysteresis was detected in both enzyme- and NADPH-started reactions. The shape of these reaction tracings makes trimethoprim appear to be a slow binding inhibitor. However, trimethoprim is not a slow-binding inhibitor when assayed under conditions that eliminate hysteresis. Contrary to this, sulfamethoxazole did not affect hysteresis or augment inhibition of the enzyme by trimethoprim. Sulfamethoxazole alone (at 6 mM) did not inhibit the enzyme. A simple procedure has been developed to circumvent hysteresis and allow reliable determinations of K_i values of both weak and tight binding inhibitors. For example, K_i values for pyrimethamine, trimethoprim, and methotrexate were found to be 214 nM, 1.3 nM, and 0.021 nM, respectively.

Dihydrofolate reductase (EC 1.5.1.3) is the site of action of several important drugs including methotrexate, trimethoprim, and pyrimethamine (Hitchings & Burchall, 1965). Presently, there is much interest in understanding the mechanisms by which these inhibitors bind to their target enzyme with such high affinity. The X-ray crystal structures of the *Escherichia coli* enzyme–methotrexate complex (Matthews et al., 1977) and the *Lactobacillus casei* enzyme–methotrexate–NADPH complex (Matthews et al., 1978) revealed many residues that intereact with methotrexate, but there is no satisfactory explanation that completely accounts for its greatly enhanced binding compared to dihydrofolate (Hood & Roberts, 1978). In fact, there is mounting evidence that the binding of ligands to dihydrofolate reductase is a complex process, and a variety of protein conformations with different binding affinities have been detected by NMR, fluorescence, and kinetic techniques (Feeney et al., 1977; Dunn et al., 1978; London et al., 1979; Williams et al., 1979). However, kinetic analyses can be difficult to perform and interpret. Both substrates are fairly unstable and can degrade to a number of products (Blakley, 1969; Lowry et al., 1961). Background (nonenzymic) changes in 340-nm absorbance are common (Baccanari, 1978), and most inhibitors of interest have low K_i values (1 nM or less). Several years ago, Jackson et al. (1977) studied methotrexate inhibition of the enzyme from several mammalian sources and measured its slow off-rate by a variety of means. More recently the general theory of tight-binding inhibitors has been expanded by Cha (1975, 1976) to include analysis of slow-binding and slow, tight-binding inhibitors (Williams & Morrison, 1979). This type of inhibition involves a two-step inhibitor binding process which includes the slow isomerization of one enzyme conformation to another and is characterized by nonlinear reaction velocities. Williams et al. (1980), studying the enzyme from *Streptococcus faecium*, noted that inhibition by several compounds, including trimethoprim and *p*-aminobenzoyl glutamate, was time dependent and interpreted these data to indicate the compounds were slow-binding inhibitors. In this study, we report conditions under which hysteresis (a slow transient velocity) is observed with uninhibited *E. coli* dihydrofolate reductase and examine its relationship to inhibitor binding. We also present a method of circumventing hysteresis and determining tight-binding K_i values.

Materials and Methods

Trimethoprim and pyrimethamine were obtained from Burroughs Wellcome Co., Greenville, NC. Sulfamethoxazole (a product of Roche) was also obtained from Wellcome. The various NADPH preparations were from Sigma Chemical Co. and represented many different "lots" manufactured over a 2-year period. We also tested NADPH from P-L Laboratories and Boehringer Mannheim. Folic acid was purchased from Calbiochem and NADP was from Boehringer Mannheim. All

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other proteins, reagents, and inhibitors were from Sigma. Dihydrofolate was prepared by the method of Blakley (1960) and stored at −70 °C in 5 mM HCl and 50 mM 2-mercaptoethanol. Aliquots of this were thawed daily and diluted with 0.1 M imidazole–HCl, pH 7, and mercaptoethanol to a stock concentration of 2.5 mM dihydrofolate in 0.6 M mercaptoethanol. Protein concentrations were determined by the method of Lowry et al. (1951) and by the fluorescent methotrexate titration of Perkins & Bertino (1966). The NADPH dissociation constant was determined by using the fluorescence method previously described (Baccanari et al., 1981).

Ultrogel AcA 54 (LKB) column chromatography was used to determine if the apparent molecular weight (elution volume) of dihydrofolate reductase varied in the presence of NADPH or as a function of the protein concentration. The column (1.5 × 21 cm) was calibrated with dextran blue, bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000), soybean trypsin inhibitor (M_r 21 500), and cytochrome *c* (M_r 12 384) at a flow rate of 10 mL/h. Dihydrofolate reductase was run under the following conditions: (1) 220 nM enzyme loaded (0.3 mL); (2) 210 nM enzyme plus 60 μM NADPH loaded, column equilibrated with 60 μM NADPH; (3) 26 nM enzyme plus 60 μM NADPH loaded, column equilibrated with 60 μM NADPH.

Enzyme Sources. The majority of work in this study was performed with the form 1 isozyme of *E. coli* RT 500 dihydrofolate reductase (Baccanari et al., 1977). The enzyme was purified to homogeneity by the methods previously described (Baccanari et al., 1981) and stored at a protein concentration of 1 mg/mL in 20 mM potassium phosphate buffer, pH 7, 2 mM dithiothreitol, and 1 mM EDTA. For the daily assays, working solutions were prepared by diluting aliquots of the stock into 0.1 M imidazole–HCl, pH 7, 2 mM dithiothreitol, and 1 mM EDTA containing 50 μg/mL bovine serum albumin. Wild-type *E. coli* B (ATCC 11303) and *E. coli* K12 (C600, pIE028, obtained from Sheila Smith, Wellcome Research Laboratories) were grown, harvested, and lysed as previously described (Baccanari et al., 1977; Smith et al., 1979). The dihydrofolate reductases from both these strains were partially purified by ammonium sulfate fractionation and gel filtration column chromatography on Sephadex G-75.

Enzyme Assays. Dihydrofolate reductase activities were determined by using a spectrophotometric assay that measures the decrease in 340-nm absorbance occurring as NADPH and dihydrofolate are converted into product. Assays were performed at 30 °C in a Gilford Model 250 recording spectrophotometer at 0.1 or 0.2 full-scale sensitivity. A chart speed of 0.5 cm/min was used to record the progress curve of the 10–20-min reaction. The standard substrate concentrations were 45 μM dihydrofolate and 60 μM NADPH in a final volume of 1 mL. One enzyme unit is defined as the amount of enzyme required to reduce 1 μmol of dihydrofolate/min based on a molar extinction coefficient of 12.3×10^3 (Hillcoat et al., 1967). Assays were performed in either buffer A [50 mM Tris-HCl, pH 7.2, 50 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA (Poe et al., 1972)], 0.1 M imidazole–HCl, pH 7, or 50 mM potassium phosphate, pH 7. The measured velocities were corrected for nonenzymic changes in 340-nm absorbance. These background rates were linear and less than 0.001/min.

Four different methods were used to initiate the assay. In method 1, all the reactants except one were added to the cuvette and preincubated in a 30 °C water bath for 2 min. The cuvettes were then transferred to the spectrophotometer (also maintained at 30 °C), and the reaction was started by adding a small volume (usually 20 μL) of the missing reactant. The final enzyme concentration in these assays was between 0.2 and 0.9 nM. In method 2, cuvettes containing 45 μM dihydrofolate and 60 μM NADPH in 0.9 mL of buffer were prewarmed at 30 °C and then transferred to the spectrophotometer. In a separate tube, the enzyme (2–9 nM in 1 mL) was preincubated with 60 μM NADPH for 2 min at 30 °C, and then a 0.1-mL aliquot was diluted 10-fold into the reaction mixture to start the assay. Method 3 is similar to method 2 except that inhibitor (at a concentration 10-fold greater than its desired final concentration) was preincubated with the enzyme and NADPH. The reaction was started by diluting a 0.1-mL aliquot of the enzyme–inhibitor–NADPH complex into the assay mixture which contained only buffer, NADPH, and dihydrofolate. Assay method 4 is another modification of method 2 and was specifically used to demonstrate the rapid association of trimethoprim to the enzyme–NADPH complex. The enzyme, 3 nM, was preincubated with 60 μM NADPH for 5 min at 30 °C, and then a small volume of trimethoprim was added (final concentration, 5 nM) and 100 μL of the resulting solution was quickly diluted 10-fold into a prewarmed assay mixture containing dihydrofolate, NADPH, and trimethoprim so that their final concentrations were 45 μM, 60 μM, and 5 nM, respectively.

It is difficult to evaluate the instantaneous velocity of curving reaction tracings "by hand" because of the errors inherent in drawing tangents to the curve. This problem was circumvented by coupling the spectrophotometer to a minicomputer which read the photometer voltage output every 50 ms. This output was averaged over 2 s, converted to an absorbance value, and stored. In processing these data, it was assumed that the velocity was essentially constant for short time intervals and was calculated by using linear regression of 1-min segments of the absorbance vs. time readings. The error of the calculated rates was almost always less than 1%.

NADPH Purification. NADPH concentrations were measured by using UV absorption spectroscopy and enzymic oxidation. The spectrophotometric determinations were made at pH 7 using molar extinction coefficients of 14.9×10^3 and 6.2×10^3 at 259 and 340 nm, respectively (Horecker & Kornberg, 1948). Enzymic oxidations were performed by incubating 12 μM NADPH and 100 μM dihydrofolate with 0.4 unit of dihydrofolate reductase in 1 mL of 0.1 M imidazole–HCl, pH 7. The concentration of NADPH oxidized (and dihydrofolate reduced) was calculated from the net change in 340-nm absorbance and the molar extinction coefficient of 12.3×10^3 (Hillcoat et al., 1967). Both methods give essentially identical results.

Several preparations of NADPH were further purified by ion-exchange column chromatography. Whatman DE-23 was converted to its bicarbonate form, packed in a 1 × 30 cm column, and thoroughly washed with 2 mM NH_4HCO_3, pH 10. NADPH (15 mg in 1 mL of the same buffer) was loaded and then eluted with a linear 2–500 mM NH_4HCO_3 gradient (1000-mL total volume) at a flow rate of 40 mL/h. One major 340-nm absorbing peak was observed, and all fractions with a 260:340 ratio of 2.3:2.4 were tested with dihydrofolate reductase.

NADPH was also prepared enzymically by incubating 1.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 6.8 units/mL glucose-6-phosphate dehydrogenase in 2 mL of 0.1 M imidazole–HCl pH 7.3, at room temperature for 10 min. The change in 340-nm absorbance indicated that greater than 90% of the $NADP^+$ was reduced by this procedure. An Amicon

Centriflow ultrafiltration membrane was used to separate glucose-6-phosphate dehydrogenase from the reactants.

A procedure was designed to remove small amounts of a presumptive tight-binding inhibitor that may be contaminating commercial NADPH. It was based on the assumption that if a potent inhibitor was present, it would bind to dihydrofolate reductase and could be separated from the remaining NADPH as the enzyme–inhibitor complex. Two different separation techniques were used. In one case, 90 μM NADPH and 1 nM enzyme (in a final volume of 1 mL) were incubated at room temperature for 10 min and then ultrafiltered by using an Amicon DM-5 membrane. The filtrate was essentially free of enzymic activity and used as a source of NADPH for the hysteresis studies. In another case, 500 μM NADPH and 6 nM enzyme (in a final volume of 5 mL) were incubated at 30 °C for 5 min and then loaded on a 2.5 × 10 cm Sephadex G-25 column and eluted with buffer A at a flow rate of 1 mL/min. The enzyme and NADPH were sufficiently separated such that the trailing half of the NADPH was devoid of all enzymic activity and used in the hysteresis studies.

K_i Determinations. Assay method 3 was used to perform inhibitor binding analyses. For these studies the 0.1 M imidazole–HCl, pH 7, assay buffer contained 2 mM ascorbate and 1 mM EDTA to reduce nonenzymic background changes in 340-nm absorbance. The final enzyme concentration in the assay was ~0.8 nM and resulted in a relatively high uninhibited velocity. For this reason the control rate was measured (usually in six to eight separate determinations) at 0.2 full-scale sensitivity over a 2–3-min time period. In the inhibited reactions, sufficient drug was used to give 80–95% inhibition, and these velocities were measured at 0.1 full-scale sensitivity over a 15–20-min time period. The inhibited reactions were linear throughout this time interval except for methotrexate and aminopterin. In these cases, only the final steady-state velocities were employed. The data were evaluated by the method of Henderson (1973) for tight-binding competitive inhibitors:

$$\frac{I_t}{1 - v_i/v_0} = K_i(1 + S/K_s)\frac{v_0}{v_i} + E_t \qquad (1)$$

where I_t is the total inhibitor concentration, v_0 is the velocity of the uninhibited reaction, v_i is the inhibited velocity, K_i is the inhibitor constant, S is the concentration of competing substrate, dihydrofolate (45 μM), K_s is the dihydrofolate Michaelis constant (8.9 μM), and E_t is the total enzyme concentration. Plots of $I_t/(1 - v_i/v_0)$ vs. v_0/v_i result in straight lines with slopes of K_i $(1 + S/K_s)$ and ordinate intercepts of E_t. Data were analyzed by using the QUADFIT program of Henderson (1973) or, when the program failed to converge, linear regression. Since the dependent variable (v_i) appears in both abscissa and ordinate terms, linear regression does not "fit" the data. However, the quality of the data was such that the experimental points fell on an obvious line and regression was primarily used as a tool to objectively and reproducibly generate a slope term. Hand fitting gave essentially the same K_i as QUADFIT or linear regression.

The method of Jackson et al. (1977) was used to calculate the dissociation rate constant (k_{off}) of methotrexate from the enzyme–methotrexate–NADPH ternary complex. The nonlinear reaction tracings generated in assay method 3 were processed by using the minicomputer described above.

Results

Nonlinear Reaction Velocities. The shape of the velocity tracings observed with *E. coli* dihydrofolate reductase varied with the order of substrate addition (assay method 1). Enzyme- or NADPH-started reactions showed essentially linear velocities throughout a 340 nm absorbance change of at least 0.05.[1] However, dihydrofolate-started reactions were nonlinear (Figure 1A). The initial velocity was greatly inhibited and then slowly increased before reaching an apparent steady state after 12–14 min. Its final velocity was ~70% of that of the enzyme- or NADPH-started reactions, even after identical total absorbance changes. This type of slow enzyme response to a rapid change in ligand concentration (in this case addition of the second substrate) has been termed hysteresis by Frieden (1970). The hysteretic response can be evaluated by expressing the amount of curvature as the ratio of initial velocity (v_0) to final velocity (v_f) or by determining the rate at which velocity changes. The hysteresis data in Figure 1A were replotted in Figure 1B as log $(v_f - v_t)$ vs. time (where v_t is the velocity at time t) and showed first-order kinetics with a rate constant of 0.2 min^{-1} and a corresponding half-time of 3.5 min.

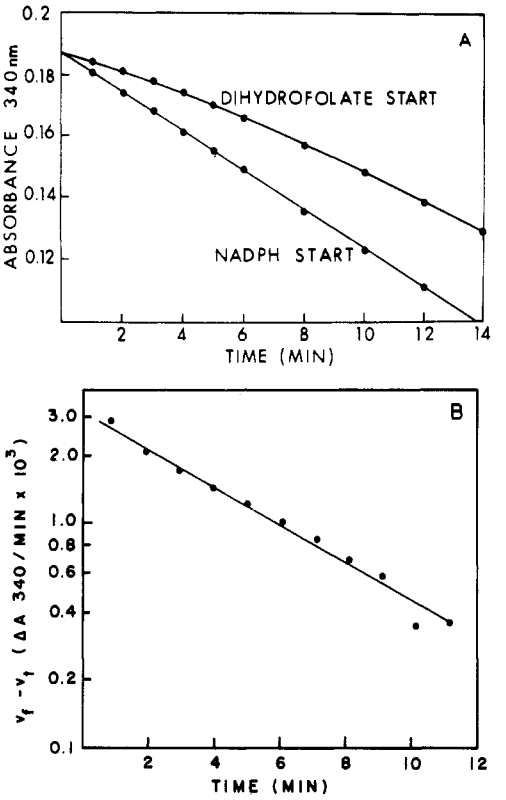

FIGURE 1: *E. coli* dihydrofolate reductase hysteresis. (A) Progress curves of dihydrofolate- and NADPH-started assays. The enzyme (0.4 nM) was preincubated at 30 °C for 2 min with all reactants except either dihydrofolate or NADPH, and then the assay was started by adding the missing component. Details are given under Materials and Methods. (B) Determination of the first-order rate constant for hysteresis of the dihydrofolate-started reaction. $K = -2.3 \times$ slope.

The hysteretic E–NADPH complex did not form instantaneously. If enzyme and NADPH were mixed and the reaction immediately started by the addition of dihydrofolate, there was no hysteresis, and the resulting linear velocity was identical with that of an enzyme- or NADPH-started reaction.

[1] The decreasing velocity observed after a total 340-nm absorbance change of 0.05–0.07 is due to substrate depletion and product inhibition. For example, with an extinction coefficient of 12.3 × 10^3 (Hillcoat et al., 1967), a change in absorbance of 0.1 corresponds to 8.1 μM substrate being converted to product. The initial velocity of a reaction mixture composed of 37 μM dihydrofolate, 52 μM NADPH, 8 μM tetrahydrofolate, and 8 μM $NADP^+$ was 83% of that of the standard reaction mixture composed of 45 μM dihydrofolate and 60 μM NADPH.

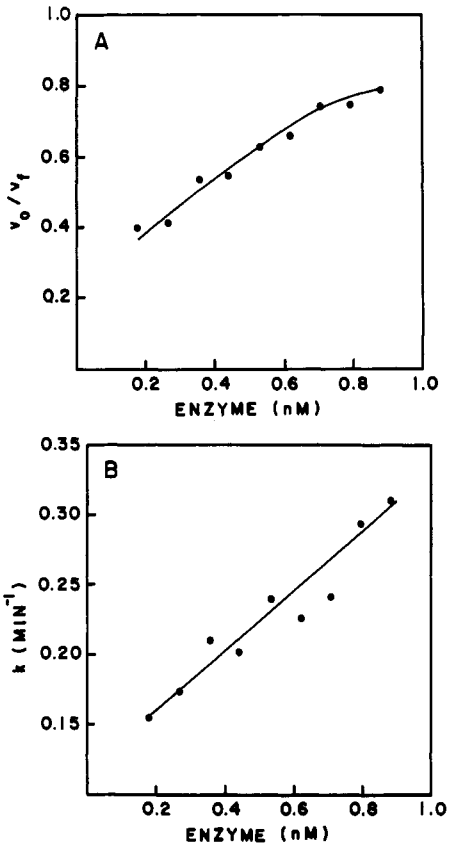

FIGURE 2: Effect of enzyme concentration on hysteresis. (A) Extent of curvature, determined by the ratio of initial velocity (v_0) to final velocity (v_f), as a function of enzyme concentration. (B) Variation of the first-order rate constant (determined as in Figure 1B) as a function of enzyme concentration.

However, increasing hysteresis was evident as enzyme and NADPH were preincubated from 0.1 to 0.8 min. Maximum hysteresis was observed by ~1 min and did not change for preincubations up to 30 min (the longest time tested).

The degree of hysteresis was affected by the buffer. Comparing three common dihydrofolate reductase assay buffers, curvature was greater in 50 mM potassium phosphate, pH 7, and in buffer A than in 0.1 M imidazole–HCl, pH 7. Two of these buffers were used in this study. The general properties of hysteresis were examined in buffer A, whereas inhibitor analyses were performed in imidazole. The rate of hysteresis was also affected by temperature. When a series of assays were performed at decreasing temperatures using sufficient enzyme to give identical final velocities in each case, the half-life of hysteresis was 4.1, 6.0, and 9.1 min at 30, 25, and 20 °C, respectively. Although these and the following studies were performed with homogeneous form 1 dihydrofolate reductase from *E. coli* RT 500, similar hysteretic responses were noted with the enzyme from "wild-type" *E. coli* B and K-12.

Effect of Enzyme Concentration. Figure 2A shows that NADPH-induced hysteresis (represented by v_0/v_f) varied with the concentration of enzyme. Hysteresis was most prominent at 0.2 nM enzyme (the lower limit of sensitivity), and the amount of curvature decreased as the enzyme concentration in the assay was increased to 1.0 nM. The rate of transition from less active to more active enzyme species also increased with enzyme concentration (Figure 2B), with half-times changing from 4.5 to 2.2 min at the two extremes. Hysteresis was evident (though diminished in magnitude) at enzyme concentrations between 1 and 2 nM but was difficult to quantitate because the fast catalysis caused significant substrate depletion and product inhibition before the steady-state velocity was attained. However, a two-step procedure was used to show that incubation of higher enzyme concentrations with NADPH completely eliminated hysteresis. For example, 10 nM enzyme preincubated with 60 μM NADPH and then diluted 10-fold to a final enzyme concentration of 1.0 nM in the assay resulted in linear reaction tracings. The velocity of this assay was identical with that of an equivalent reaction (1.0 nM enzyme) started with enzyme that had not been preincubated with NADPH. Therefore, hysteresis and the dependence of reaction velocity on the order of reagent addition were enzyme concentration dependent and completely eliminated at "high" enzyme levels. Under these latter conditions, hysteresis could not be made to reappear by increasing the preincubation NADPH concentration, preincubation time, or both. As shown below, preincubations at high enzyme concentrations will serve as the basis for inhibitor K_i determinations.

Enzyme concentration dependent hysteresis is often associated with reversible enzyme polymerization with the various polymers having different kinetic properties (Frieden, 1970). However, no evidence of polymerization was detected with these preparations of *E. coli* dihydrofolate reductase. Polyacrylamide electrophoresis of the enzyme in the presence or absence of $NaDodSO_4$ and amino acid sequence analysis show that the enzyme is a monomer with a molecular weight of ~18 000 (Baccanari et al., 1977). Also, gel filtration chromatography showed that the enzyme elution volume or shape of the elution profile did not change in columns equilibrated with buffer A or buffer A plus 60 μM NADPH or when the enzyme concentration applied to the column was varied between 220 and 26 nM so that the peak elution concentrations were either 21 or 1 nM (see Materials and Methods). Therefore, there was no evidence of enzyme aggregation under conditions which showed hysteresis in the assay.

Effect of NADPH. Several years ago, Williams et al. (1977), working with the *L. casei* enzyme, noted nonlinear reaction tracings that were characterized by a short burst of activity preceeding a slower linear velocity. Neither the order of reagent addition nor their concentrations had any effect on the extent of curvature. Rather, it was shown to be dependent upon the source and age of commercial NADPH and was considered to be an artifact caused by minor but potent inhibitory impurities in the coenzyme preparations. Even though the properties of the nonlinear *E. coli* dihydrofolate assays differed from those of the *L. casei* enzyme, the possible effect of NADPH impurities on hysteresis was examined further. *E. coli* dihydrofolate reductase hysteresis was not dependent upon any particular batch or commercial source of NADPH. It was observed with DEAE-cellulose column chromatography purified NADPH and in samples freshly reduced from $NADP^+$ (see Materials and Methods). The possibility that NADPH was contaminated by a small amount of an ubiquitous, tight-binding inhibitor was also tested by incubating the NADPH with enzyme, separating the E–NADPH (and presumably the E–I) complex from the bulk of the coenzyme, and then testing the resulting NADPH for hysteresis. The separations were performed by either Ultrogel AcA 54 column chromatography or Amicon ultrafiltration (see Materials and Methods), and in both cases the "enzyme-scrubbed" NADPH showed the same degree of hysteresis as the starting material.

Figure 3 shows the relationship between hysteresis and the concentration of NADPH. Curvature of the assay increased with the preincubation concentration of NADPH until the maximum effect was observed at ~100 nM coenzyme. Increasing the NADPH concentration to 76 μM had no addi-

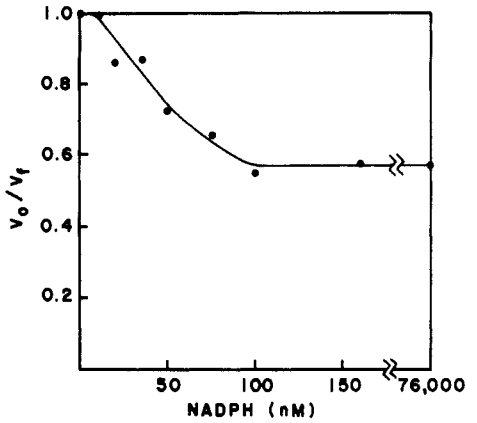

FIGURE 3: Hysteresis as a function of NADPH concentration. The enzyme (0.4 nM) was preincubated with various concentrations of NADPH, and then the reaction was started by adding dihydrofolate plus additional NADPH so that the final coenzyme concentration was 76 μM.

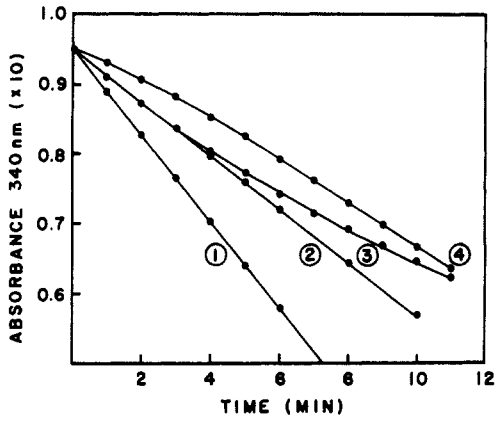

FIGURE 4: Effect of inhibitors on hysteresis. Assays were performed by using method 1 and contained ~0.4 nM enzyme. (1) NADPH-started reaction, no inhibitor. (2) Enzyme was preincubated with NADPH and 1 μM pyrimethamine, and the reaction was started with dihydrofolate. (3) Enzyme was preincubated with dihydrofolate and 5 nM trimethoprim, and the reaction was started with NADPH. (4) Enzyme was preincubated with NADPH and 5 nM trimethoprim, and the reaction was started with dihydrofolate.

tional effect. The half-time of these responses appeared independent of NADPH concentration and had an average value of 3.6 min. The concentration of NADPH showing half-maximal curvature (50 nM) was somewhat less than the fluorometrically determined equilibrium dissociation constant (250 nM) measured in the same buffer.

Effect of Inhibitors. Pyrimethamine and trimethoprim had markedly different effects on the shape of the dihydrofolate reductase reaction tracings (Figure 4). Line 1 shows a normal, uninhibited, NADPH-started reaction maintaining a constant velocity throughout the assay period. The dihydrofolate-started control assay is not shown but is similar to that illustrated in Figure 1. Line 4 shows a trimethoprim-inhibited, dihydrofolate-started reaction. Hysteresis is evident with a final, inhibited steady state attained after 6–8 min. However, the trimethoprim-inhibited, NADPH-started reaction (line 3) was inhibited, and then the rate further decreased throughout the assay period and did not obtain steady state. Additional information on trimethoprim binding is presented under Inhibitor Analysis. Contrary to this, pyrimethamine changed the shape of the dihydrofolate-started reaction from hysteretic to linear (line 2). The pyrimethamine-inhibited, NADPH-started reaction also appeared linear and had the same activity as line 2 (data not shown).

Previously, sulfa drugs were reported to inhibit *E. coli* dihydrofolate reductase and potentiate the inhibition of trimethoprim (Poe, 1977). However, in our hands, 6 mM sulfamethoxazole did not inhibit the enzyme from *E. coli* RT 500, *E. coli* B, or *E. coli* K-12 and did not affect hysteresis or alter inhibition of the enzyme by trimethoprim.

Inhibitor Analysis. E. coli dihydrofolate reductase activity is adversely affected by small inorganic cations. Its turnover number and affinity for NADPH and methotrexate decrease in buffers containing sodium or potassium ions (Baccanari et al., 1981). For this reason, salt-free 0.1 M imidazole–HCl, pH 7, is the buffer of choice for inhibitor analysis in this laboratory. However, as with buffer A, the shapes of the trimethoprim-inhibited reaction tracings in imidazole buffer were dependent upon the order of reagent addition, making it difficult to quantitate trimethoprim binding. For example, by use of assay method 1, the trimethoprim I_{50} value[2] was calculated to be 8.1 nM from the initial velocities of the dihydrofolate-started reactions, whereas a value of 5.1 nM was obtained from the final velocities of the same reaction tracings. An I_{50} value of 9.8 nM was calculated from the initial velocities of equivalent NADPH-started reactions, though a value could not be determined from their final velocities because these (inhibited) reactions did not reach steady state during the 15-min assay.

Although the time-dependent shapes of the trimethoprim-inhibited velocity tracings are similar to those seen with slow-binding inhibitors (Williams et al., 1980), they are also similar to the hysteresis observed in the absence of inhibitors and could represent augmented hysteresis rather than slow binding. Evidence supporting this latter possibility was gained by performing inhibitor analyses using an assay procedure (method 3) similar to that which eliminated hysteresis. When the enzyme was preincubated with NADPH and trimethoprim and then diluted 10-fold into the assay mixture, the initial velocities observed were inhibited and linear throughout the 10–15-min assay period. During preincubation, the enzyme–trimethoprim–NADPH complex was formed in the absence of dihydrofolate. After dilution, a new (lower) steady-state concentration of the complex was attained in the presence of the competing substrate, dihydrofolate. The linear, inhibited velocities observed indicated that dissociation of trimethoprim from the enzyme–trimethoprim–NADPH complex was not slow and that steady state was attained in the 4–5-s interval between initiating and recording the reaction. Similarly, by using assay method 4, it was possible to show that the binding of trimethoprim to the enzyme–NADPH complex was also rapid. Under these conditions, trimethoprim was added to the enzyme–NADPH complex and an aliquot was immediately diluted into the assay mixture. The linear, inhibited velocities observed indicated that association of trimethoprim with the enzyme–NADPH complex was not slow and that steady state was attained in the 4–5-s interval between adding trimethoprim and recording the reaction. Therefore, under these conditions, trimethoprim is not a slow-binding inhibitor.

Assay method 3 was adopted as the preferred manner of measuring inhibited velocities, and K_i values were calculated from these data by using the procedure of Henderson (1973). Trimethoprim and pyrimethamine were found to have K_i values of 1.2 nM and 214 nM, respectively. The trimethoprim value is in close agreement with the "overall" inhibition constant (K_i*) of 0.96 nM determined by Williams et al. (1980)

[2] The I_{50} values reported for the hysteretic reactions have been corrected for depletion of free inhibitor by using Cha's (1975) equation for competitive inhibitors: $I_{50} = K_i(1 + S/K_s) + {}^1/_2E_t$.

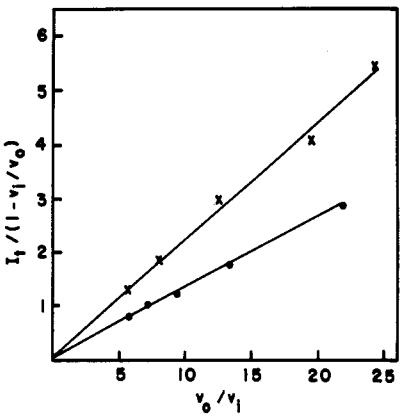

FIGURE 5: Henderson plot of methotrexate inhibition. The enzyme (0.65 nM final concentration) was assayed by using method 3 at various methotrexate concentrations (between 0.6 and 5.2 nM). K_i values, calculated with 45 μM dihydrofolate (●) or 90 μM dihydrofolate (×), are 22.7 pM and 19.9 pM, respectively.

for the *S. faecium* enzyme. When assay method 3 was used with methotrexate, nonlinear reaction tracings were again observed. In this case, the initial rates were slow and then increased throughout a 5–20-min period before attaining a final (inhibited) steady-state rate. Since methotrexate is a tight-binding inhibitor, this lag actually reflects the slow dissociation of inhibitor from the enzyme–methotrexate–NADPH complex that precedes dihydrofolate binding in its steady-state proportion. The kinetic method described by Jackson et al. (1977) was used to calculate the methotrexate rate constant for dissociation (0.012 min^{-1}, corresponding to a half-time of 56 min). This is similar to the value of 0.0138 min^{-1} reported for the L1210 enzyme (Jackson et al., 1977). Figure 5 shows an analysis of the steady-state, methotrexate-inhibited velocities at two dihydrofolate concentrations. The crossing lines on the ordinate are indicative of competitive inhibition and the calculated K_i value was 21 pM. Aminopterin, another tight-binding dihydrofolate reductase inhibitor, also showed slow dissociation and its calculated K_i value was 37 pM.

Discussion

The transients observed in the *E. coli* dihydrofolate reductase assay can best be described in terms of the hysteresis concept. According to Frieden (1970), hysteretic enzymes are those which respond slowly (usually in terms of reaction velocity) to a rapid change in ligand concentration. Most hysteresis mechanisms involve at least two enzyme forms with different kinetic properties and the slow conversion of one to the other during the enzyme assay. In the present case, the hysteretic complex (E′–NADPH) was formed when enzyme (E) was incubated with NADPH and then, upon the addition of dihydrofolate, there was an apparent slow conversion from an inactive (or low-activity) complex to an active enzyme species (E–NADPH). The simplest type of mechanism which usually describes these results is

$$\begin{array}{l} \text{E + NADPH} \rightleftharpoons \text{E–NADPH} \overset{\text{slow}}{\rightleftharpoons} \text{E′–NADPH} \\ \quad\quad + \\ \quad\quad FH_2 \\ \quad\quad \downarrow \\ \quad\quad \text{E + products} \end{array}$$

mechanism I

In the present case about 100 nM NADPH was required for maximal hysteresis, and time studies indicated that the conversion of E–NADPH to E′–NADPH was complete within 1 min at 30 °C. The regeneration of active enzyme in the full reaction system followed a first-order process, and the rate constants were temperature dependent. Since the enzyme- or NADPH-started reactions were linear and had higher velocities than comparable dihydrofolate-started reactions, it appeared that E′ was not a natural component of the enzyme sample and could not be generated in the presence of dihydrofolate.

In most well-characterized hysteresis systems, the conversion from inactive to active enzyme species involves displacement of a tightly bound ligand, a conformational isomerization, or a polymerization/depolymerization reaction (Ainslie et al., 1972; Frieden, 1979). However, in the present case, the nature of E′ is unknown. The possibility that hysteresis is the result of a tight-binding inhibitor in the NADPH preparations was tested, and no evidence was found to support this hypothesis. There is evidence from a variety of sources that dihydrofolate reductases can undergo conformational changes upon the binding of NADPH (Matthews et al., 1978, and references cited therein) but the enzyme concentration dependency of hysteresis (Figure 2) argues against E′ being a simple conformer of E. Decreasing hysteresis with increasing enzyme concentration is usually indicative of a polymerization step with the monomer being the inactive (E′) enzyme species, but *E. coli* dihydrofolate reductase is not an oligomeric enzyme. Aggregated forms have been detected but only at high protein concentrations (~10 mg/mL) or following enzyme sulfhydryl group oxidation (Baccanari et al., 1975). The monomer has always been considered to be the enzymatically active species. In the present study, there was no evidence for molecular weight changes when gel filtration column chromatography was performed in the presence or absence of NADPH at protein concentrations comparable to those used in the enzyme assay.

In the past, other dihydrofolate reductases were shown to have unusual kinetic properties. For example, Dann et al. (1976) reported that the *L. casei* enzyme had nonlinear progress curves under some (unspecified) assay conditions, and Kaufman & Gardiner (1966) noted unexplained rate accelerations during assays with the chicken liver enzyme. More recently, NADPH-induced changes in the kinetics of methotrexate binding have been reported for the L1210 dihydrofolate reductase (Fan et al., 1980). In this study, we observed hysteresis with a "wild-type" *E. coli* B and an *E. coli* K-12 enzyme in addition to the *E. coli* RT 500 enzyme. Therefore, there is evidence that hysteresis is a property of *E. coli* dihydrofolate reductase in general and may be found with the dihydrofolate reductases from other sources.

Recently, Williams et al. (1980) noted that inhibition of the *S. faecium* dihydrofolate reductase by trimethoprim was time dependent and interpreted this as "slow-binding" inhibition. There was an excellent correlation between the experimental and theoretical data. The *E. coli* enzyme also showed qualitatively similar behavior with trimethoprim (Figure 4) in that, with NADPH-started reactions, trimethoprim binding was so slow that steady state was not reached during the 15-min assay. However, in this case, the results are not sufficient to characterize trimethoprim as a slow-binding inhibitor, because under different assay conditions (assay method 4), steady state was attained in less than the 5 s required to initiate the reaction. As far as the *E. coli* enzyme is concerned, more needs to be known about the mechanism of hysteresis and the conformational states generated by NADPH before we can use the shape of the reaction tracing to support general conclusions about inhibitor binding mechanisms. On the other hand, quantitation of inhibitor binding affinities (using assay method 3) is reliable and appears to be independent of the effect that any particular inhibitor has on hysteresis. For example, py-

rimethamine eliminated hysteresis whereas trimethoprim augmented it (assay method 1), yet both gave linear responses in the K_i assay. Under these conditions, only methotrexate and aminopterin showed nonlinear reaction tracings, and this reflects the slow dissociation of the tight-binding inhibitors from the enzyme. The only anomaly we encountered with the K_i assay was that the graphically determined enzyme concentration (y intercept) was consistently less than the "known" concentration. One of the more extreme examples of this is shown in Figure 5 where the y intercept of 0.15 nM must be contrasted with the 0.65 nM value calculated by using fluorescent methotrexate titrations and a molecular weight of 18 000. Although this discrepancy could be related to the observation that the enzyme appeared to exist as an active polymer in the hysteresis studies, no conclusions have been reached. More importantly, we found that this discrepancy did not compromise the quality of the data. In all cases, setting the y intercept at the "known" enzyme concentration only changed the calculated K_i by 5–10% (data not shown).

In 1976, Poe (1977) reported that sulfamethoxazole and a number of other sulfa drugs are inhibitors of *E. coli* MB 1428 dihydrofolate reductase and presented evidence that they potentiate the inhibition of trimethoprim. The proposed mechanism of potentiation is the simultaneous binding of trimethoprim and sulfa drug to the enzyme, and it was suggested that this potentiation is responsible for the synergism observed when the drugs are used together "in vitro" as antibacterials. This novel hypothesis was contested on both theoretical and practical grounds (Burchall, 1977; Then, 1977). We attempted to examine sulfamethoxazole inhibition of the *E. coli* enzyme to determine if hysteresis was responsible for the reported potentiation, but the drug (at a concentration of 6 mM) did not inhibit any of the *E. coli* dihydrofolate reductases tested. It also did not increase the inhibition of a fixed level of trimethoprim or change the shape of the trimethoprim-inhibited velocity tracing. It is unlikely that these discrepancies are a result of using enzyme from different *E. coli* strains, because the *E. coli* MB 1428, RT 500, and K-12 enzymes have similar primary amino acid structures (Smith & Calvo, 1980). It is important to note that in our initial studies some sulfamethoxazole samples did moderately inhibit the enzyme, with 50% inhibitory concentrations of 1–3 mM. However, when these tests were repeated with four different batches of pharmaceutical-grade sulfamethoxazole, no inhibition was observed. We feel that the slight inhibition we originally saw was probably due to contamination of the sulfamethoxazole by a small amount (probably in the range of 1–2 ppm) of one of the tight-binding dihydrofolate reductase inhibitors commonly used in our laboratory.

Overall, these studies illustrate some of the complexities of the *E. coli* dihydrofolate reductase enzyme assay and offer practical methods for obtaining consistent kinetic data.

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