Mechanism of Hysteresis in Bovine Glutamate Dehydrogenase: Role of Subunit Interactions†

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ABSTRACT: Hysteresis in glutamate dehydrogenase is observed only in the reductive amination reaction and only with GTP present. The rate of reductive amination with NADH as coenzyme increases during the time course of the reaction. Premixing experiments, where glutamate dehydrogenase is preincubated with various combinations of substrates and GTP, suggest that the hysteresis phenomenon is not due to a time-dependent conformational change in the enzyme. Enzyme dilution experiments show (i) that the hysteresis is not due to enzyme association–dissociation effects and (ii) that the onset of the activation occurs after accumulation of about 25 μM NAD^+. Addition of NAD^+ to the initial reaction mixture prevents hysteresis from occurring. Although with NADPH as coenzyme hysteresis does not occur, addition of $NADP^+$ to initial reaction mixtures containing NADH blocks hysteresis. A model based on reciprocating subunits is proposed whereby hysteresis results from product (NAD^+) accumulation resulting in a half-of-the-sites activation of reductive amination.

Ox liver glutamate dehydrogenase (GDH) (EC 1.4.1.3) catalyzes the oxidative deamination of L-glutamate and a variety of other amino acids (Struck & Sizer, 1960). The enzyme is unique among mammalian dehydrogenases in utilizing either NAD(H) or NADP(H) with comparable efficacy (Engel & Dalziel, 1969). In the direction of oxidative deamination, the enzyme exhibits negative homotropic interactions (negative cooperativity) which have been attributed to induced conformational changes occurring in an enzyme–NAD(P)$^+$–glutamate ternary complex (Dalziel & Engel, 1968). Evidence from binding studies (Dalziel & Egan, 1972) and from studies of induced conformational changes (Bell & Dalziel, 1973) supported such a model. That such conformational changes occur in the catalytic cycle has recently been demonstrated (Alex & Bell, 1980). In the reductive amination reaction, no such effects have been observed (Engel & Dalziel, 1970). In the presence of GTP, however, it has been observed that the rate of reductive amination increases during the time course of the reaction. This effect has been termed hysteresis (Frieden, 1970) and has been attributed to slow conformational changes occurring in the protein conformation in the presence of NADH and GTP, leading to a form of the enzyme that has an increased rate of reductive amination.

During the course of our continuing studies on the mechanism and regulation of glutamate dehydrogenase, we have reexamined the phenomenon of hysteresis exhibited by this enzyme. On the basis of the studies reported here, we propose a molecular mechanism for the hysteresis phenomenon exhibited by glutamate dehydrogenase that is fundamentally and conceptually different from previous models. This model is based on the concept of reciprocal interactions between the subunits of glutamate dehydrogenase rather than on time-dependent conformational changes.

Materials and Methods

Bovine liver glutamate dehydrogenase used in these experiments was obtained as a crystalline suspension in ammonium sulfate from Sigma Chemical Co. Prior to use, the enzyme was thoroughly dialyzed against 0.1 M sodium phosphate, pH 7.0, containing 10 μM ethylenediaminetetraacetic acid (EDTA). Enzyme concentrations were measured spectrophotometrically by using an extinction coefficient at 280 nm of 0.93 cm^{-1} for a 1 mg/mL solution (Egan & Dalziel, 1971). Concentrations of enzyme used in reaction mixtures are calculated by using an active site molecular weight of 56 000.

Buffer salts, substrates, GTP, and the coenzymes NAD^+, $NADP^+$, NADH, and NADPH were obtained from Sigma Chemical Co. All coenzymes were assayed for purity enzymatically by using either glutamate dehydrogenase, lactate dehydrogenase, or alcohol dehydrogenase. All coenzymes used were of greater than 98% enzymatic purity. Rates of reductive amination were determined spectrophotometrically with a Cary 219 recording spectrophotometer. Rates of NAD(P)H oxidation were calculated by using a millimolar extinction coefficient of 6.22 cm^{-1} at 340 nm (Stein et al., 1963). Data are represented either as direct spectrophotometer tracings of OD_{340} vs. time, as plots of incremental rate vs. elapsed reaction time, where tangents to the OD_{340} vs. time curves are taken at 10-s intervals and the calculated rates at these time points plotted vs. elapsed time, or as the ratio of the initial rate to the highest incremental rate obtained for a particular set of conditions.

Results

Hysteresis Phenomenon: Preincubation Experiments. Initially experiments were performed over a range of substrate (α-ketoglutarate and ammonium chloride) and coenzyme concentrations in the presence and absence of GTP to establish optimal conditions for observing hysteresis. The results of these experiments established that hysteresis was most markedly observable with 0.1 mM NADH, 0.8 mM α-ketoglutarate, 20 mM ammonium chloride, and in the presence of 0.05 mM GTP. In the absence of GTP, hysteresis effects were not observed. Under the above conditions, a series of preincubation experiments were performed where enzyme was preincubated with various combinations of substrates, coenzymes, and GTP, with reaction being initiated by addition of the omitted reactant. These conditions are given in Table I. In all cases, identical extents of hysteresis were observed.

Experiments with Varied Enzyme Concentrations. Since glutamate dehydrogenase is known to undergo a reversible, concentration-dependent aggregation (Frieden, 1959a,b), the effects of enzyme concentration on the hysteresis phenomenon were studied. Enzyme concentration was varied over an 8-fold range with 0.1 mM NADH, 0.8 mM α-ketoglutarate, 20 mM

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Table I: Enzyme-Ligand Preincubation Experiments

premixed reactants	reaction initiated by addition of	observation
NH_4^+, αKG, GTP, enzyme [a]	NADH	hysteresis (1.27) [b]
NH_4^+, αKG, GTP, enzyme	αKG	hysteresis (1.27)
enzyme, NADH, NH_4^+	GTP + αKG	hysteresis (1.27)
NADH, αKG, NH_4^+, GTP	enzyme	hysteresis (1.27)
NADH, αKG, GTP, enzyme	NH_4^+	hysteresis (1.27)

[a] αKG, α-ketoglutarate. [b] Ratio of initial incremental rate to the highest observed incremental rate—a value of 1.0 indicates no hysteresis, with values greater than 1.0 indicating hysteresis.

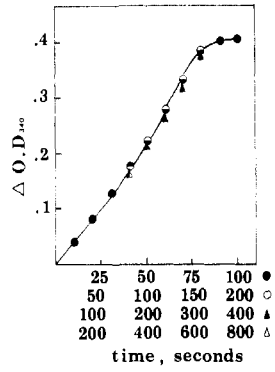

FIGURE 1: Effects of enzyme concentration on the hysteresis phenomenon. Plots of ΔOD_{340nm} vs. time with enzyme concentrations of 0.64 (●), 0.32 (○), 0.16 (▲), and 0.08 μM (△) are shown. The following time axes were used: (●) 0 → 100 s; (○) 0 → 200 s; (▲) 0 → 400 s; (△) 0 → 800 s. Other conditions were 0.8 mM α-ketoglutarate, 100 μM NADH, 20 mM ammonium chloride, 50 μM GTP, and 0.1 M sodium phosphate buffer, pH 8.0.

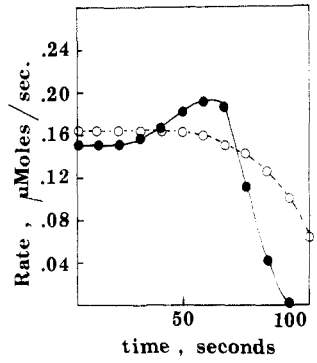

FIGURE 2: Comparison of NADH and NADPH with regard to hysteresis. Plots of incremental rate vs. elapsed time with NADH (●) and NADPH (○) as coenzyme are shown. Other conditions were as described in Figure 1 except with 0.64 μM glutamate dehydrogenase.

ammonium chloride and 0.05 mM GTP, and the rates of reductive amination were followed. The results, shown as direct OD_{340} vs. time plots in Figure 1, show that over this range of enzyme concentrations the hysteresis phenomenon is observed in each case. As the enzyme concentration is decreased, however, the time for appreciable acceleration of the reaction was increased. In all cases, hysteresis was not observed until approximately 25% of the initially added NADH was utilized.

Experiments with NADPH as Coenzyme. In addition to using NADH as coenzyme in these experiments, we have also used NADPH as substrate. As is shown in Figure 2 when NADH and NADPH were compared under similar conditions with respect to α-ketoglutarate, ammonium chloride, and GTP concentrations, no hysteresis effects with NADPH as coenzyme were observable. Various concentrations of NADPH were tested, but in all cases, hysteresis was not observed.

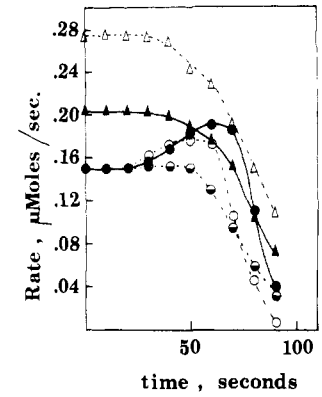

FIGURE 3: Effects of NAD^+ concentration on the hysteresis phenomenon observed with NADH as coenzyme. Plots of incremental rate vs. elapsed time with NAD^+ concentrations of (●) 0, (○) 0.1, (◒) 0.4, (▲) 2.4, and (△) 6 mM are shown. Other conditions are as described in Figure 2.

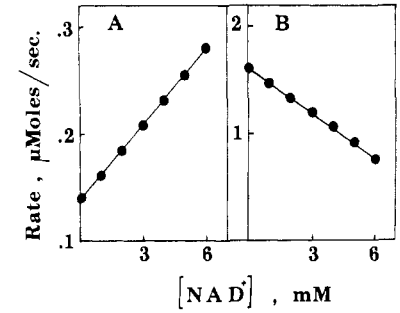

FIGURE 4: Effects of NAD^+ concentration on initial rates of reductive amination with NADH as coenzyme. Plots of initial rate vs. NAD^+ concentration (A) with 50 μM GTP and (B) with no GTP present are shown. Other conditions were 0.8 mM α-ketoglutarate, 100 μM NADH, 20 mM ammonium chloride, and 0.1 M sodium phosphate buffer, pH 8.0, with 0.64 μM GDH.

Effects of Oxidized Coenzymes. In light of the premixing experiments described earlier, we have examined the effects of addition of oxidized coenzymes on hysteresis effects. Figure 3 shows the effects of increasing NAD^+ concentrations on hysteresis as represented in plots of incremental rate vs. elapsed time. The following two effects are seen: (a) the hysteresis effect is diminished as the concentration of NAD^+ increases; at NAD^+ concentrations of 2.0 mM and above, *no* hysteresis is seen, and (b) the rate of reductive amination by NADH is not appreciably affected by concentrations of NAD^+ up to 1 mM, but above this concentration, NAD^+ *activates* the reductive amination utilizing NADH. As is shown in Figure 4A, the rate of reductive amination increases as the concentration of NAD^+ increases even up to 6 mM NAD^+, the highest NAD^+ concentration used. When these experiments are repeated in the absence of GTP (that is, under conditions where no hysteresis is observed), NAD^+ acts, as expected, as an inhibitor of reductive amination (Figure 4B).

Similar experiments were performed with NADPH as coenzyme in the presence and absence of GTP (Figure 5) with NAD^+ as the inhibitor. Also shown in Figure 5 are the effects of addition of $NADP^+$ to reductive amination with NADPH as coenzyme. However, when the effects of $NADP^+$ addition to NADH reaction mixtures are studied (in the presence of GTP; i.e., under hysteresis conditions), it is found (Figure 6) that the hysteresis response was blocked. When the concentration dependence of these oxidized coenzyme effects on the hysteresis observed with NADH as coenzyme (in the presence of GTP) is studied, it is found that both NAD^+ and $NADP^+$ block hysteresis over a comparable concentration range.

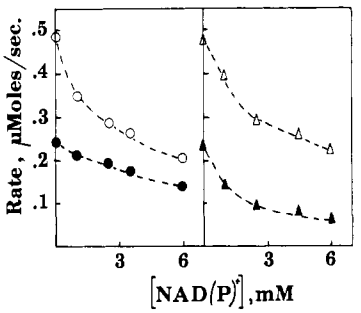

FIGURE 5: Effects of oxidized coenzymes on initial rates of reductive amination with NADPH as coenzyme. Plots of initial rate vs. $NAD(P)^+$ concentration with 100 μM NADPH, 0.8 mM α-ketoglutarate, and 20 mM ammonium chloride in the presence (closed symbols) and absence (open symbols) of 50 μM GTP are shown with (●, ○) NAD^+ and (▲, △) $NADP^+$ as inhibitors.

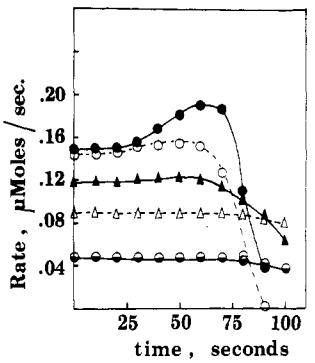

FIGURE 6: Effects of $NADP^+$ concentration on the hysteresis phenomenon observed with NADH as coenzyme. Plots of incremental rate vs. elapsed time with NADH as coenzyme are shown. (●) No $NADP^+$; (○) 0.1 mM $NADP^+$; (▲) 0.4 mM $NADP^+$; (△) 1.0 mM $NADP^+$; and (⊖) 6 mM $NADP^+$.

Discussion

That glutamate dehydrogenase exhibits hysteretic phenomena is not a new observation. Frieden and co-workers (Frieden, 1970; Bates & Frieden, 1973; Frieden & Colman, 1967) have described a hysteretic displacement of GTP inhibition and changes in the state of aggregation of the enzyme. The acceleration of the rate of reductive amination with NADH as coenzyme, which is observed only in the presence of GTP, appears to fall into the class of hysteretic displacement of GTP inhibition. It is the molecular mechanism of this phenomenon which will be discussed here.

Since the hysteresis phenomenon is observed only with NADH and not with NADPH, it is probably related to binding of NADH to a second, regulatory site to which NADPH cannot bind. Frieden (1959a,b) originally proposed that such a regulatory site for NADH but not NADPH exists. Subsequently, many binding studies of reduced coenzymes [for a review, see Dalziel (1975)] have established that this is indeed the case. Both NADH and NADPH bind at the active site, exhibiting negative cooperativity (George & Bell, 1980), while NADH binds to a second site per subunit and NADPH will bind at this additional site with greatly decreased affinity, and under the conditions used here may be considered to bind only at the active site. In the presence of GTP, strong inhibition of reductive amination with NADH is observed (Frieden, 1963; Huang & Frieden, 1972), and binding studies have shown that the binding of GTP and NADH is strongly synergistic (Frieden, 1965; Pal & Colman, 1979). The hysteresis phenomenon with glutamate dehydrogenase has been attributed to slow conformational changes occurring in an enzyme complex containing NADH and GTP. The premixing studies described here (summarized in Table I) suggest either that a slow conformational change is not responsible for the acceleration of reductive amination or that such conformational change occurs only in the presence of *all* of the substrates for reductive amination and GTP, i.e., in the active quaternary complex. The enzyme dilution experiments, shown in Figure 1, however, suggest that the phenomenon is not associated with a time-dependent conformational change in an enzyme complex but rather with accumulation of NAD^+. By varying the enzyme concentration over an 8-fold range under otherwise identical conditions, it is apparent that the hysteresis phenomenon is occurring, but over widely different time scales with different enzyme concentrations. In each case, the onset of the acceleration phase of the reaction occurs only after about 25–30 μM NAD^+ has been accumulated. This observation led us to a series of experiments in which oxidized coenzyme (i.e., product) is added to the initial reaction mixture. As shown in Figure 3, when increasing NAD^+ concentrations are added to the initial reaction mixture, little inhibition of reductive amination with NADH as coenzyme is seen; however, as the NAD^+ concentration is increased, the acceleration of reductive amination is considerably reduced, and at NAD^+ concentrations of 1 mM, it is no longer observed. Above about 2 mM NAD^+, the reductive amination reaction is actually activated, as seen in Figure 4A. When these experiments are repeated in the absence of GTP, only inhibitory effects of NAD^+ are observed (Figure 4B). Similar experiments (Figure 5) with NADPH in the presence or absence of GTP with either NAD^+ or $NADP^+$ as added oxidized coenzyme show only inhibitory effects. The activation effects at high NAD^+ concentrations are observed only with NADH and GTP. It appears that at high concentrations NAD^+ is mimicking the effects of ADP and reversing the GTP inhibition of reductive amination. ADP effectively reverses GTP inhibitory effects as has been shown previously (Wolff, 1962; Frieden, 1963). Clearly the activatory effects of NAD^+ under these conditions are quite separate from the effect of blocking the hysteresis phenomenon. In fact, when low concentrations of ADP are added to initial reaction mixtures containing NADH and GTP, the reaction rate is activated, but hysteresis effects are still observed.

Perhaps the most significant observation in terms of the molecular mechanism of the phenomenon described here is the fact that $NADP^+$ has similar effects on the acceleration phase of the reductive amination reaction with NADH as coenzyme as does NAD^+ (Figure 6) and removes the acceleration phase over the same concentration range as does NAD^+.

While the phenomenon described here is clearly a time-dependent process, it appears to be due to a time-dependent accumulation of NAD^+ rather than to a slow conformational change. The accumulated NAD^+ clearly can relieve the potent GTP inhibition of reductive amination with NADH as coenzyme. As such, this process is not really a true hysteretic phenomenon as recently defined (Neet & Ainslie, 1980), and an alternative mechanism to explain these results must be found. Several alternative mechanisms can be proposed and these are discussed below.

Second Site Hypothesis. Each subunit in the hexamer has an active site, which can bind either NAD(H) or NADP(H). In addition, there is a second site which binds NADH, but not NADPH, and sites for GTP and ADP which are overlapping (Prough et al., 1973; Frieden, 1965). In the presence of GTP, the enzyme is strongly inhibited due to binding of NADH but not NADPH to the second site. A simple hypothesis would be that substrate depletion during the reaction reduces the

NADH concentration to such an extent that the second, inhibitory site is no longer liganded, causing an apparent activation. While such a scheme could explain the lack of acceleration with NADPH as substrate (it cannot bind to the second site, and hence, there is nothing to activate during the time course of the reaction) and could conceivably explain the effect of NAD^+ in blocking the acceleration (by direct competition with the inhibitory site), several points argue against this simple model. First, the phosphorylated coenzymes do not bind to this second site; second, the oxidized coenzyme blocking of the acceleration effect is seen at $NAD(P)^+$ concentrations of less than 1 mM, and direct binding studies of oxidized coenzymes have shown only binding at the active site under such conditions (Dalziel & Egan, 1972); finally, competitive binding at the inhibitory site would be expected to lead to activation; with both NAD^+ and $NADP^+$, no activation is observed at concentrations where the acceleration phenomenon is blocked. NAD^+, as discussed earlier, does activate at higher concentrations by mimicking ADP. Incidentally, this activation seen at high NAD^+ concentrations does suggest that NAD^+ can bind to more than one site per subunit. The binding studies of Dalziel & Egan (1972) extended to a total coenzyme concentration of 2 mM; the activation effects of NAD^+ are seen at higher concentrations. This observation lends weight to the recent suggestion based on CD spectral simulation studies (Bayley & O'Neil, 1980) that there might be a second NAD^+ site per subunit. The present studies show that, similar to the binding of reduced coenzymes, $NADP^+$ does not bind to this second site.

Reciprocal Interactions between Subunits. Since the above models involving a second site effect of oxidized coenzymes can be eliminated, the only binding site available for oxidized coenzyme binding to elicit its effects on the acceleration of reductive amination with NADH as coenzyme is the active site. In order to explain such a model, reciprocal interactions between subunits within the hexamer must be invoked. To explain the observations reported here, one must consider that in the enzyme hexamer functioning in the reductive amination reaction not all of the active sites are liganded by NADH. Since NAD(P)H binding to the active site shows negative cooperativity in both the presence and absence of substrate analogues (George & Bell, 1980), it is a reasonable conjecture that three of the six sites may be operating in reductive amination (i.e., a half-of-the-sites type reaction). In the presence of GTP, the reductive amination reaction is strongly inhibited by NADH binding to a second, inhibitory site per subunit. When externally added NAD^+ or $NADP^+$ is added, it binds to the unliganded active sites and blocks the hysteresis-type phenomenon from taking place. Since the only site per subunit which can bind both NAD^+ and $NADP^+$ is the active site, the similarity of the effects of externally added NAD^+ or $NADP^+$ shows that it is to this site they are binding and blocking the hysteresis-type phenomenon. Direct binding studies of oxidized coenzymes (Dalziel & Egan, 1972) have shown that in the absence of a glutamate analogue, NAD^+ binding has a K_D of about 0.5 mM and apparently binds to only three sites per hexamer. This dissociation constant, and the number of binding sites per hexamer, is quite consistent with the apparent affinity of $NAD(P)^+$ in producing these effects (an approximate dissociation constant of 0.5 mM is obtained from the concentration dependence of these effects) and the idea of a half-of-the-sites type effect).

Clearly such a model explains the effects of added NAD^+ or $NADP^+$ blocking the hysteresis-like acceleration of reductive amination. However, in this form, the model does not account for the acceleration phenomenon observed for reductive amination with NADH as coenzyme in the presence of GTP. A modification of this simple half-of-the-sites model is required. The concept of reciprocating subunits (or flip-flop enzymes) is not new, having been first proposed for alkaline phosphatase (Lazdunski et al., 1971). Such models suggest that, in the steady state, half of the active sites are occupied by reactants and half by products. In the case of glutamate dehydrogenase, the accumulation of NAD^+ which results in the acceleration of reductive amination by using NADH in the presence of GTP can be interpreted as requiring the concomitant presence of both NAD^+ *and* glutamate in half of the active sites. This produces a conformational change in the active sites catalyzing the reductive amination reaction that results in the relief of the inhibition produced by NADH binding, in the presence of GTP, to the inhibitory site. With such a model, NAD^+ or $NADP^+$ alone would not be expected to produce an activation, merely block the hysteresis-like phenomenon. This requirement for the concomitant presence of NAD^+ and glutamate to produce activation is not unreasonable since several other studies have shown that NAD^+ binding to three out of the six active sites per hexamer in the presence of glutarate (an analogue of glutamate) induces a conformational change in the oligomer (Chen & Engel, 1974; Bell & Dalziel, 1973), and in one of these studies (Bell & Dalziel, 1973), it has been shown that the conformational change occurs across subunit interfaces. It might also be noted that in the presence of glutarate, the affinity for NAD^+ of the first three sites per hexamer liganded is comparable to what would be expected on the basis of the amount of NAD^+ that accumulates prior to the acceleration phase becoming noticeable.

In addition to an explanation of the effects of exogenously added $NAD(P)^+$ on the acceleration of reductive amination with NADH as coenzyme in the presence of GTP and the acceleration phase of the time course itself, the above model provides a rationale for the lack of inhibition seen with exogenously added $NAD(P)^+$. Since added $NAD(P)^+$ will bind to active sites which are not required for the reductive amination reaction and shows apparent negative cooperativity in binding to the remaining sites in the hexamer, direct competition with NADH on the "reductive amination" active sites is not expected. The slight inhibition of reductive amination by NAD^+ and $NADP^+$ presumably results from spillover from the "$NAD(P)^+$ sites". It should be noted that the above considerations apply only to the GTP-inhibited form of the enzyme. However, because of the high affinity of the enzyme for GTP and the synergistic effects of GTP and coenzyme binding, this form of the enzyme may well be a physiologically significant form.

In summary, it is apparent that the observations reported here can most simply be explained in terms of a reciprocating subunit model for the GTP-inhibited form of glutamate dehydrogenase and that such a model is consistent with previous studies of ligand binding and induced conformational changes in glutamate dehydrogenase.

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Inactivation of Dihydrofolate Reductase from *Lactobacillus casei* by Diethyl Pyrocarbonate†

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ABSTRACT: The role of histidine residues of dihydrofolate reductase from *Lactobacillus casei* was investigated with diethyl pyrocarbonate. This enzyme has no cysteine residues and differs in this respect from many nicotinamide nucleotide dehydrogenases, which have catalytically important sulfhydryl groups. X-ray studies of this enzyme have shown that histidine residues are involved in substrate binding but not in proton transfer [Matthews et al. (1978) *J. Biol. Chem. 253*, 6946]. Dihydrofolate reductase was inactivated by diethyl pyrocarbonate; the second-order rate constant for the reaction was 29 M^{-1} min^{-1} at 0 °C. The difference spectrum of native and diethyl pyrocarbonate inactivated enzyme had a maximum near 242 nm, which indicated a reaction with histidine residues. The absence of any spectral difference near 280 nm indicated that diethyl pyrocarbonate had not reacted with tyrosine residues. Dihydrofolate reductase lost all of its enzymatic activity after about six of the seven histidine residues had been modified. No catalytic activity was lost during an initial rapid reaction with about four histidine residues, but a subsequent slower reaction involving an additional one or two residues was associated with the loss of activity. The enzyme was protected from inactivation by either of the substrates NADPH or dihydrofolate. In fact, treatment with diethyl pyrocarbonate in the presence of either substrate, but particularly with NADPH, resulted in substantially greater activity than that found with untreated enzyme. Treatment with 1 M hydroxylamine partially restored activity to dihydrofolate reductase that had been inactivated by diethyl pyrocarbonate.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate by NADPH to yield 5,6,7,8-tetrahydrofolate, an essential cofactor in the biosynthesis of thymidylate and purines. Antifolate compounds such as methotrexate, aminopterin, pyrimethamine, and trimethoprim form stable complexes with dihydrofolate reductase and presumably owe their chemotherapeutic activities to the coincident inhibition that ultimately results in decreased DNA biosynthesis.

X-ray studies have shown that dihydrofolate reductases from both *Lactobacillus casei* and *Escherichia coli* have quite similar three-dimensional structures (Matthews et al., 1977, 1978). However, their amino acid sequences are different, though some regions have a high degree of homology (Stone et al., 1977; Freisheim et al., 1978). Many nicotinamide nucleotide dehydrogenases have catalytically essential sulfhydryl groups; a sulfhydryl group in the dihydrofolate reductase from *E. coli* is essential in maintaining an active conformation (Williams & Bennett, 1977), whereas the enzyme from *L. casei* has no cysteine residues. Histidine residues have been shown to be catalytically essential in a number of nicotinamide nucleotide dehydrogenases, where their proposed function is as a proton donor and acceptor (Dalziel, 1975). No histidine residue is involved in proton transfer in dihydrofolate reductase from either *L. casei* or *E. coli* (Matthews et al., 1978), but histidine residues are involved in substrate binding to the *L. casei* enzyme (Birdsall et al., 1977; Matthews et al., 1978, 1979) and in maintaining an active conformation of the *E. coli* enzyme (Greenfield, 1974; Williams, 1975). Two histidine residues of dihydrofolate reductase of *E. coli* react rapidly with diethyl pyrocarbonate, resulting in a loss in catalytic activity (Greenfield, 1974). There is little if any correspondence between the position of the seven histidines in the amino acid sequence of the *L. casei* enzyme and the five histidines in the *E. coli* enzyme (Freisheim et al., 1978). Despite the structural similarities between the *E. coli* and *L. casei* enzymes, they differ in a number of respects that might

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