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CARBODIIMIDE-DEPENDENT INACTIVATION OF DIHYDROFOLATE REDUCTASE

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SUMMARY: Dihydrofolate reductase from amethopterin-resistant Lactobacillus casei was inactivated by a water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl. The rapid inactivation observed at pH 5.0-6.0, coupled with lack of recovery of activity from inactivated samples incubated with NH_2OH was consistent with modification of enzymic carboxyl groups. Significant protection against inactivation was provided by 7,8-dihydrofolate and NADPH. Analysis of the reaction order suggests that the carbodiimide-dependent inactivation may result from modification of a single essential carboxyl group.

Dihydrofolate reductase, an NADPH-dependent enzyme that reduces H_2folate[2] to (-)H_4folate, serves as a major site for the chemotherapeutic action of amethopterin and trimethoprim. Its low molecular weight (~20,000) and facile isolation from numerous sources permit detailed examination of the enzyme by a variety of chemical and physical methods, including fluorescence, circular dichroism, UV, and nuclear magnetic resonance spectroscopy. Chemical modification studies of tryptophan with N-bromosuccinimide (1,2), arginine with phenylglyoxal (3), and lysine with dansyl chloride (4) or 2,4-pentanedione (5) implicate the side chains of these residues in the active site of dihydrofolate reductase from amethopterin-resistant Lactobacillus casei. The three dimensional structure of the L. casei enzyme-NADPH-amethopterin ternary complex, solved at 2.5 Å resolution, demonstrates that the substrate and coenzyme binding functions are carried out by overlapping

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2. Abbreviations used: H_2folate, 7,8-dihydrofolate; (-)-H_4folate, 5,6,7,8-tetrahydrofolate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; MES, 2(N-morpholino)ethane sulfonic acid; Hepes, N-2-hydroxethylpiperazine-N-ethanesulfonic acid; Pipes, 1,4-piperazinebis(ethanesulfonic acid).

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segments of the amino acid sequence (6). Further, in addition to ascribing particular roles for tryptophan, lysine, and arginine residues in coenzyme and inhibitor interactions with the enzyme, the results of X-ray analysis indicate the involvement of other amino acid residues in binding and catalytic roles. For example, on the basis of the close interaction of the side chain of Asp-26 with N-1 of amethopterin in the L. casei ternary complex, Matthews et al. (6) proposed that this carboxyl group is crucial to the extremely strong binding of amethopterin and may serve as a proton source in the enzymic reaction. The Asp-26 is conserved in all bacterial reductases sequenced to date and is replaced by Glu in all animal reductases examined thus far (7,8). As a means of testing the latter hypothesis, we have initiated studies of the chemical modification of dihydrofolate reductase with water-soluble carbodiimides, which are reagents known to exhibit selectivity for reaction with carboxyl groups in proteins (9-11).

METHODS AND MATERIALS: Dihydrofolate reductase was purified to homogeneity from an amethopterin-resistant strain of L. casei (1) and was judged to be pure by analytical polyacrylamide gel electrophoresis. Enzyme activity was determined spectrophotometrically on a Beckman Acta CV spectrophotometer by measuring the decrease in absorbance at 340 nm due to the disappearance of both H_2folate and NADPH. Protein concentrations were determined using a molar extinction coefficient of 26,400 $M^{-1}cm^{-1}$ at 278 nm (1). The assay solution for dihydrofolate reductase contained 50 µl 2.5 x 10^{-3}M H_2folate solution, 50 µl 2 x 10^{-3}M NADPH solution, and 0.9 mL of a 0.1 M potassium phosphate buffer, pH 7.5, 0.1 M KCl. H_2folate was prepared by dithionite reduction of folic acid (12)(Calbiochem) and was stored in vacuo at -70°. EDC was purchased from Sigma Chemical Company and NADPH was obtained from P-L Biochemicals. All other chemicals were reagent grade. In all cases, the extent of inactivation was determined by spectrophotometric assay of enzyme activity. The specific conditions for the chemical modification studies reported herein are provided in the legends to Figures 1-4 and Table I.

RESULTS AND DISCUSSION: The inactivation of dihydrofolate reductase in the presence of 25 and 50 mM EDC was studied as a function of the pH of the potassium phosphate buffer system (Fig. 1). The profile in Fig. 1 reveals extensive and rapid loss of activity at and below pH 6 with little or no inactivation found above pH 6.8. This behavior was indicative of carbodiimide-dependent modification of carboxyl functions which is known to proceed through intermediate formation of the corresponding O-acylisourea (9).

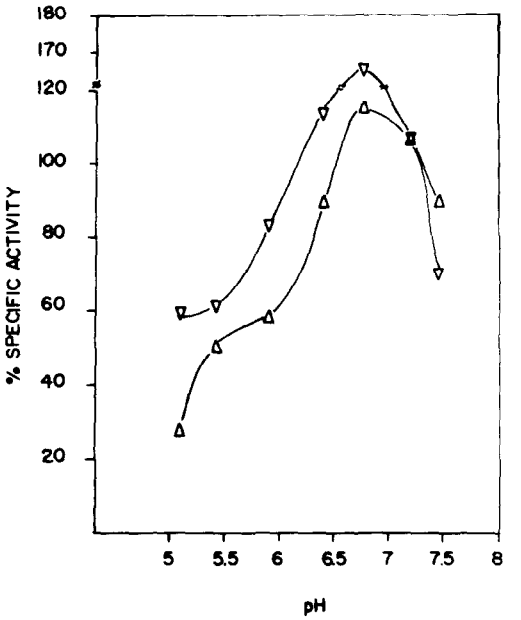

Fig. 1 Effect of pH on the rate of inactivation by EDC. Dihydrofolate reductase (5.68×10^{-5}M) was incubated at 30° with 25 (▽) or 50 (△) mM EDC in 25 mM potassium phosphate buffer at the indicated pH values. Enzyme activity was assayed after ten minutes of incubation of EDC with each sample and was referenced to the activity of control enzyme samples incubated under the same conditions but in the absence of EDC.

Although carbodiimides are highly selective for the modification of carboxyl groups at acidic pH (10,13), their reaction with enzymic cysteinyl and tyrosyl side chains is also possible under these conditions (13,14). Since L. casei dihydrofolate reductase has no cysteines (8), the only likely candidates for the rapid inactivation observed at acidic pH are carboxyl groups (14 Asp, 7 Glu and C-terminal Ala) and phenolic hydroxyl groups (5 tyrosine residues) (8). Carraway and Koshland (14) have shown that hydroxylamine reverses the reaction of carbodiimides with phenolic side chains but leaves the modification of carboxyl groups intact. Thus, dihydrofolate reductase, which had been inactivated with EDC, was dialyzed for up to six hours in buffer-containing 0.5 M hydroxylamine HCl at pH 7.0. No activity was recovered after dialysis, suggesting that the modification of tyrosyl residues was not responsible for inactivation.

To optimize modification conditions, the effect of buffer composition on the extent of inactivation by 0.1 M EDC was determined. The results in

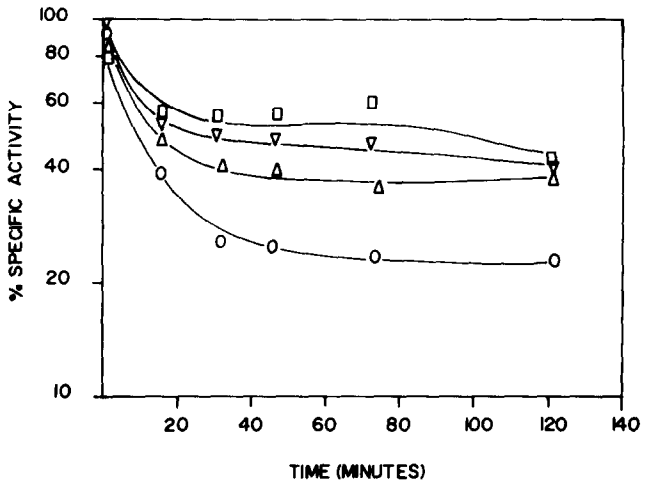

Fig. 2 Effect of phosphate concentration on inactivation by carbodiimide. Enzyme (5.68×10^{-5}M) was incubated at 30° with 50 mM EDC at pH 5.5 in potassium phosphate buffers at concentrations of 25 (○), 50 (△), 75 (▽), or 100 (□) mM. Control enzyme samples containing no EDC (not shown) lost less than 10% activity over the course of the experiment.

Table I indicate that modification in phosphate buffer was less effective relative to inactivation in Tris-HCl, MES, Hepes, and Pipes buffers. More detailed studies in phosphate buffer (Figure 2) showed that the extent of inactivation increased as the phosphate concentration decreased in the incubation mixture. In a study of enolase, George and Borders (15) observed that phosphorylated substrates depleted the active concentration of carbodiimide through condensation of the modification agent and the phosphate moiety. By comparison (see Table I), the components of the Tris-HCl, Hepes,

Table I

Inactivation of Dihydrofolate Reductase by 0.1 M EDC as a Function of Buffer Composition and pH

Buffer	pH	Percent Inactivation
0.1 M phosphate	5.5	60
0.1 M phosphate	6.0	18
0.1 M phosphate	6.5	12
0.1 M Tris Cl	5.5	80
0.1 M Tris Cl	6.0	60
0.1 M Tris Cl	6.5	38
0.1 M Hepes	5.5	82
0.1 M Pipes	5.5	80
0.1 M MES	5.5	81

Modification of enzyme (5.65×10^{-5}M) was performed for 30 minutes in the presence of 0.1 M EDC at 20° followed by assay of control and modified samples to determine extent of inactivation.

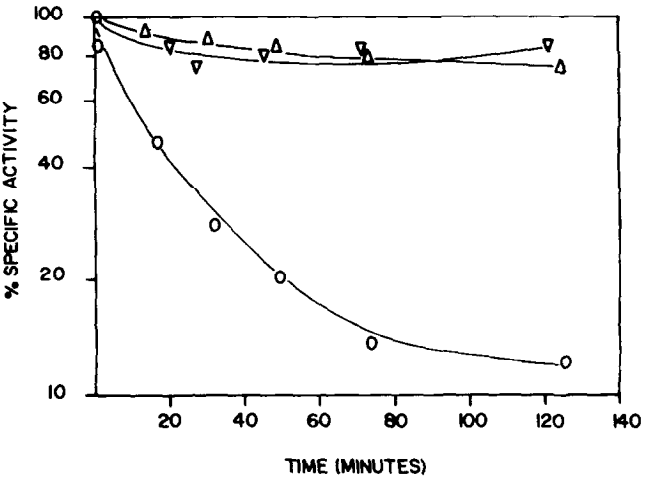

Fig. 3 NADPH and H_2folate protection of dihydrofolate reductase from inactivation by EDC. Enzyme (5.68×10^{-5}M) was incubated at 20° in 25 mM potassium phosphate buffer, pH 5.5, with a 5-fold molar excess of H_2folate (△) or a 5-fold molar excess of NADPH (▽) for 10 min. prior to making incubation mixture 0.1 M in EDC. Inactivation by 0.1 M EDC of an unprotected enzyme sample (○) is shown for comparison. Control enzyme samples containing no EDC (not shown) lost less than 10% activity over the course of the experiment

Pipes, and MES buffers did not react readily with EDC.

Incubation of dihydrofolate reductase (5.68×10^{-5}M) with a 5-fold molar excess of H_2folate or NADPH resulted in substantial protection against inactivation by 0.1 M EDC (Figure 3). After 60 min of incubation with 0.1 M EDC, the unprotected enzyme lost 88% of its original activity compared to losses of 21% and 19% in the enzyme samples protected by H_2folate and NADPH, respectively. The substrate protection observed in this experiment, where the carbodiimide was in a 350-fold molar excess over the protecting agents, clearly was not due to the depletion of active reagent as is the case with the enolase study where the concentrations of phosphorylated substrate and carbodiimide were equimolar (15).

Increasing concentrations of EDC were shown to accelerate inactivation of the enzyme (Figure 4). Using the graphical analysis employed by Levy et al. (16), the order of the EDC inactivation of dihydrofolate reductase was estimated. From initial rates of inactivation obtained with increasing concentrations of EDC, a plot (not shown) of log ($1000/t_{1/2}$) vs.

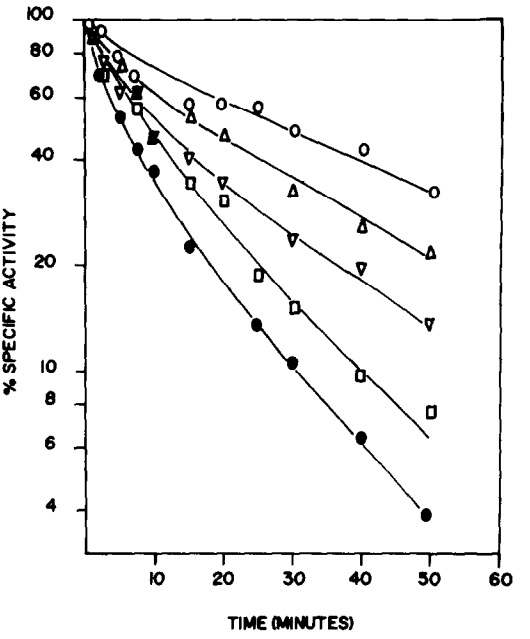

Fig. 4 Concentration dependence of carbodiimide inactivation of dihydrofolate reductase. Enzyme (5.68×10^{-5}M) was incubated at 25° in 50 mM MES buffer, pH 5.0, containing 10 (○), 17.5 (△), 25 (▽), 37.5 (□), or 50 (●) mM EDC. Enzyme incubated in the absence of EDC under these conditions lost less than 10% of its activity in one hour.

log·1000[EDC] yielded a slope of 0.84. This analysis suggested that the carbodiimide-mediated inactivation of dihydrofolate reductase approximated the reaction of EDC with a single essential enzymic carboxyl group, although EDC may have also modified other groups on the enzyme which were not involved in activity.

Studies with radiolabeled carbodiimides are planned to quantitate and identify the residue (5) whose modification by EDC was responsible for inactivation of the enzyme.

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