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MODIFICATION OF LYSYL RESIDUES OF DIHYDROFOLATE REDUCTASE WITH 2,4-PENTANEDIONE

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Summary

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) from an amethopterin-resistant strain of *Lactobacillus casei* was inactivated by 2,4-pentanedione. The inactivation appears to be due to the specific interaction of 2,4-pentanedione with lysyl residues. Inactivation is concomitant with the modification of three lysyl residues. Both NADPH and dihydrofolate protect the enzyme against inactivation, suggesting that the critical residue(s) lies at or near their binding sites. Unlike native dihydrofolate reductase, 2,4-pentanedione-modified enzyme does not form binary complexes with either NADPH, dihydrofolate or amethopterin which are stable to gel filtration. Treatment of the modified enzyme with nucleophilic reagents such as hydroxylamine, failed to promote reactivation of the enzyme. Reactivation was achieved following gel filtration at pH 6.0 and was found to be dependent on the degree to which the enzyme was inactivated.

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of L-7,8-dihydrofolate to (—)-L-5,6,7,8-tetrahydrofolate and is the target for amethopterin, a folate antagonist which is widely used in cancer chemotherapy. Dihydrofolate reductase isolated from an amethopterin-resistant strain of *Lactobacillus casei* consists of a single polypeptide with a molecular weight of 18 300 [1]. The

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N-bromosuccinimide-dependent oxidation of a single tryptophyl residue in this enzyme was well correlated with both the extent of enzyme inactivation and the loss in the capability of the enzyme to form an electrophoretically stable binary complex with NADPH [1]. Chemical modification experiments coupled with sequence analysis identified Trp-21 as the crucial tryptophan residue [2]. Comparison of the amino acid sequences for bacterial dihydrofolate reductases [3] and for the mammalian enzyme [4] reveals that Trp-21 is conserved in all of the reductases examined and that the regions surrounding this critical residue exhibit extensive homology.

Chemical modification studies have recently been directed at establishing the possible role of cationic side chains in the binding of the anionic substrates. In studies with phenylglyoxal, Vehar and Freisheim [5] have implicated two arginyl residues in the NADPH binding region while Vehar et al. [6] interpreted the results of a dansyl chloride modification of the enzyme to suggest a role for a single lysyl residue in the coenzyme binding site. In the present study the involvement of lysine in the mode of action of dihydrofolate reductase is further explored through chemical modification studies with 2,4-pentanedione, a reagent which was found to be highly selective for lysine residues in several enzymes [7—9].

Materials and Methods

Dihydrofolate was prepared by the dithionite reduction of folic acid [10]. (±)-Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid in acetic acid [11] and was stored in vacuo at —70°C [12]. NADPH was purchased from P-L Biochemicals and $NADP^+$ was obtained from the Sigma Chemical Company. 2,4-Pentanedione (99%+, Gold Label) was purchased from the Aldrich Chemical Company. [^{3}H]Amethopterin was purchased from Amersham-Searle and [^{3}H]dihydrofolate was a generous gift of Dr. Ray Cybulski (University of South Carolina). All other chemicals were reagent grade.

Enzyme purification and assay. 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 of both the native enzyme (form I) and the binary complex (form II, generated by incubating the enzyme with a 2-fold molar excess of NADPH [13]). Dihydrofolate reductase activity was determined spectrophotometrically on a Beckman Acta V spectrophotometer by measuring the decrease in absorbance at 340 nm due to the disappearance of both dihydrofolate and NADPH. The homogeneous enzyme showed specific activities in the range of 11.5—17 units/mg when assayed at pH 7.5. One unit of dihydrofolate reductase is defined as the amount of enzyme catalyzing the reduction of 1 μmol dihydrofolate/min at 25°C. The assay solution consisted of 50 μl $2.5 \cdot 10^{-3}$ M dihydrofolate solution, 50 μl $1 \cdot 10^{-3}$ M NADPH solution and 1.1 ml of a 0.1 M potassium phosphate buffer (pH 7.5), 0.1 M KCl. Protein concentrations were determined using a molar extinction coefficient of 26 400 $M^{-1} \cdot cm^{-1}$ at 278 nm [1] or by using a Bio-Rad protein assay kit.

Amino acid analysis. The amino acid analyses of dihydrofolate reductase

were performed on a Beckman Model 119C amino acid analyzer equipped with a Beckman System AA computing integrator. Samples of both native and 2,4-pentanedione-modified dihydrofolate reductase (1 mg protein) were hydrolyzed in constant boiling HCl in evacuated serum bottles (Otwell, H.B., Donato, H. and Dunlap, R.B., unpublished data) for 24 h at 110°C. The hydrolyzed samples were then dried in a heated vacuum desiccator, dissolved in buffer and applied to the analyzer.

Inactivation and protection studies. For these studies an incubation solution was prepared which had a final composition of 0.1 M potassium phosphate (pH 7.0), 0.1 M KCl, 20% glycerol and 0.2 M 2,4-pentanedione. The 2,4-pentanedione was prepared daily by diluting stock reagent with buffer to give a 1 M solution. The reaction was initiated by the addition of the enzyme at a final concentration of 0.1 mg/ml to the latter mixture which was incubated at 30°C. 20-μl aliquots were periodically withdrawn for assay. Protection studies were conducted as described above, except the substrates, products or analogues were preincubated with enzyme before addition to the reaction mixture.

Quantitation studies. Enzyme inactivated as described above was immediately subjected to chromatography on a 3 X 20 cm Sephadex G-10 column equilibrated with 0.1 M potassium phosphate, 0.1 M KCl (pH 7.0) to remove excess ligand. Samples emerging from the column were scanned from 400 to 250 nm. The enamine formed by modification of the amino groups of the enzyme molecule by 2,4-pentanedione was quantitated using an extinction coefficient of $2.0 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [9] at 312 nm. Protein concentration was estimated from the absorbance at 278 nm after the experimentally determined value was corrected for the absorbance of the enamine at this wavelength.

Ligand binding studies. Inactivated dihydrofolate reductase which was freed of excess reagent by gel filtration chromatography was concentrated to 1 mg/ml with sucrose at 4°C. The enzyme was then treated with a 2-fold molar excess of either NADPH, [^{3}H]dihydrofolate or [^{3}H]amethopterin. After 10 min incubation, excess ligand was removed by chromatography on Bio-Gel P-6. Complex formation was investigated either spectrophotometrically (for NADPH) or by counting in a Nuclear-Chicago Isocap/300 liquid scintillation counter.

Reactivation studies and stability of the enamine. Dihydrofolate reductase, which had been inactivated by 2,4-pentanedione, was applied to a 3 X 20 cm Sephadex G-10 column equilibrated with 0.1 M potassium phosphate, 0.1 M KCl (pH 7.0). Following the chromatography, fractions were made 0.1 M in either hydroxylamine, methoxyamine, pyridine-2-aldoxime methiodide, dithiothreitol or bisulfite and incubated at 30°C (pH 7.0). Similarly, enzyme which had been inactivated to different degrees was subjected to gel filtration on a 3 X 20 cm Sephadex G-10 column equilibrated with 0.1 M potassium phosphate, 0.1 M KCl (pH 6.0). Fractions containing protein were incubated at 30°C and aliquots were periodically removed for assay and spectrophotometric scans.

In order to determine the stability of the enamine, enzyme which had been inactivated to 20% of its original activity and freed of excess 2,4-pentanedione by gel filtration on Sephadex G-10 in 0.1 M potassium phosphate, 0.1 M KCl

(pH 7.0) was incubated at room temperature and was periodically scanned from 400 to 250 nm over a 16 h period.

Results

Inactivation studies

Treatment of dihydrofolate reductase with 0.2 M 2,4-pentanedione at pH 7.0 and 30°C resulted in total inactivation of the enzyme after 3 h following pseudo first-order kinetics (Fig. 1). It was observed that 20% glycerol was necessary to prevent precipitation of pentanedione-modified enzyme and that even in the presence of 20% glycerol, enzyme concentrations in excess of 0.1 mg/ml resulted in precipitation of the protein.

Quatitation of lysyl groups modified

It was observed that the extent of enamine formation was linearly related to the degree of inactivation (Fig. 2). Extrapolation to 100% inactivation indicates that complete loss of enzymatic activity correlates with the modification of 2.9 lysyl residues/enzyme molecule.

Effect of substrates and products on inactivation

Both NADPH and dihydrofolate were observed to afford significant protection against inactivation at molar ratios of about 1 : 1. More pronounced protection was observed at molar ratios of these ligands of 5 : 1, 10 : 1 and 20 : 1 (Table I). NADPH afforded better protection than did dihydrofolate, with a 20 : 1 molar ratio of NADPH to enzyme yielding complete protection against inactivation after 140 min of incubation (Fig. 1). The products $NADP^+$ and tetrahydrofolate, in contrast, offered no protection against inactivation by 2,4-pentanedione.

Exclusion of arginine modification as a basis for inactivation

Gilbert and O'Leary [7] demonstrated that 2,4-pentanedione is specific for

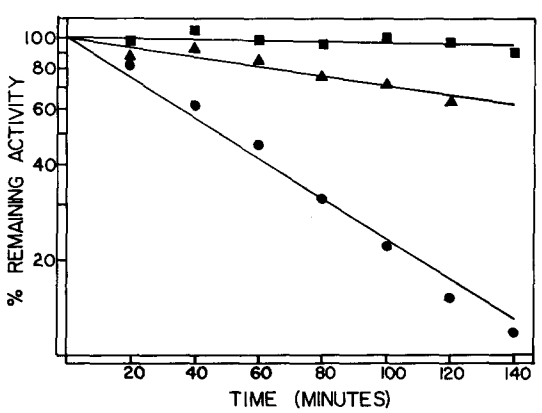

Fig. 1. Inactivation of dihydrofolate reductase (0.1 mg/ml) by 0.1 M 2,4-pentanedione in 0.1 M phosphate buffer (pH 7.0), 0.1 M KCl, 20% glycerol at 30°C. ●, enzyme alone; ■, enzyme in the presence of a 20-fold excess of NADPH; ▲, enzyme in the presence of a 20-fold excess of dihydrofolate.

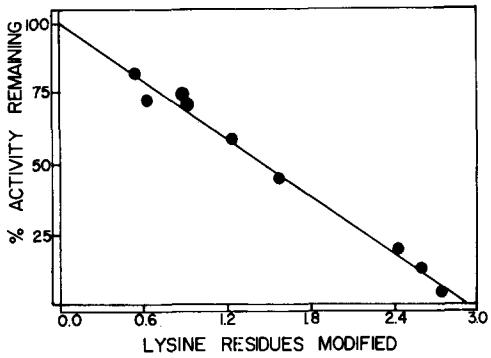

Fig. 2. Correlation of the 2,4-pentanedione-dependent inactivation of dihydrofolate reductase with the modification of lysyl residues. Modified enzyme was subjected to chromatography on Sephadex G-10 in 0.1 M potassium phosphate buffer (pH 7.0) and scanned from 400 to 250 nm.

lysine residues at pH 7.0, while arginyl residues are modified somewhat slowly even at elevated pH and buffer concentrations. A sample of enzyme which had lost 90% of its initial activity under the standard 2,4-pentanedione modification conditions was subjected to acid hydrolysis and subsequent amino acid analysis. Acid hydrolysis of 2,4-pentanedione-modified lysine results in free lysine while hydrolysis of arginine modified by the reagent results in the formation of ornithine and 2-hydroxy-4,6-dimethylpyrimidine [7]. However, asymmetry in the lysine peak on the amino acid chromatogram, indicative of ornithine, was not observed. Further, the number of arginine residues in the modified enzyme was identical to that of native dihydrofolate reductase.

Ligand binding studies

The binary complex formed between dihydrofolate reductase and NADPH

TABLE I

PROTECTION BY LIGANDS AGAINST INACTIVATION OF DIHYDROFOLATE REDUCTASE BY 2,4-PENTANEDIONE

Enzyme (0.1 mg/ml) was incubated with ligand prior to addition of 0.2 M 2,4-pentanedione. Following addition of reagent the mixture was incubated at 30°C.

Ligand	Fold excess over enzyme	% activity remaining after 140 min
NADPH	20	98
NADPH	10	90
NADPH	1	60
NADPH, (±)-tetrahydrofolate	1,1	59
Dihydrofolate	20	62
Dihydrofolate	10	48
Dihydrofolate	1	23
Dihydrofolate, $NADP^+$	1,1	22
Tetrahydrolate	20	20
$NADP^+$	20	13
2′,5′-ADP	20	13
NMN^+	20	13
NMNH	20	13
NMNH, 2′,5′-ADP	20	13
None	—	13

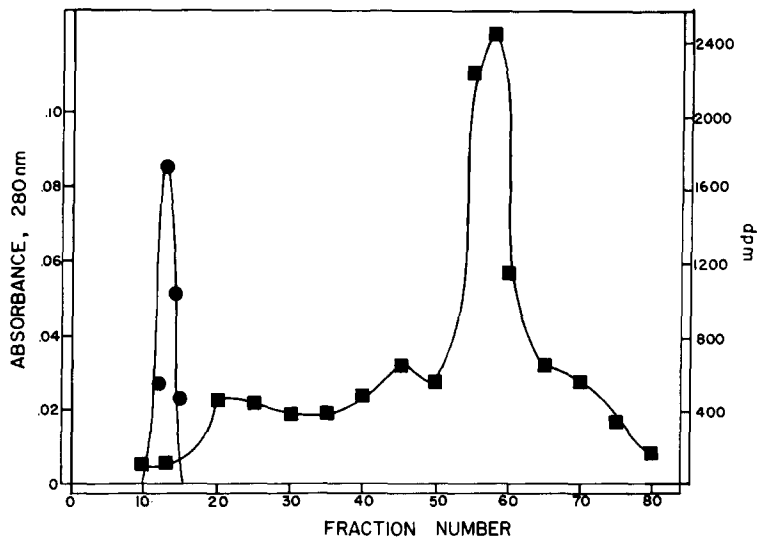

Fig. 3. Elution profile of Bio-Gel P-6 chromatography of 2,4-pentanedione-modified dihydrofolate reductase after incubation with a 2-fold molar excess of [^{3}H]dihydrofolate. 280 nm, ●; dpm, ■.

exhibits a characteristic ultraviolet spectrum with principal absorption maxima at 247 nm and 340 nm. The strong, though non-covalent, complex is stable to Bio-Gel P-6 chromatography. Addition of a 20-fold excess of NADPH to 2,4-pentanedione-modified dihydrofolate reductase resulted in the isolation of a protein exhibiting a spectrum characteristic of 2,4-pentanedione-modified enzyme and not of the enzyme-NADPH complex.

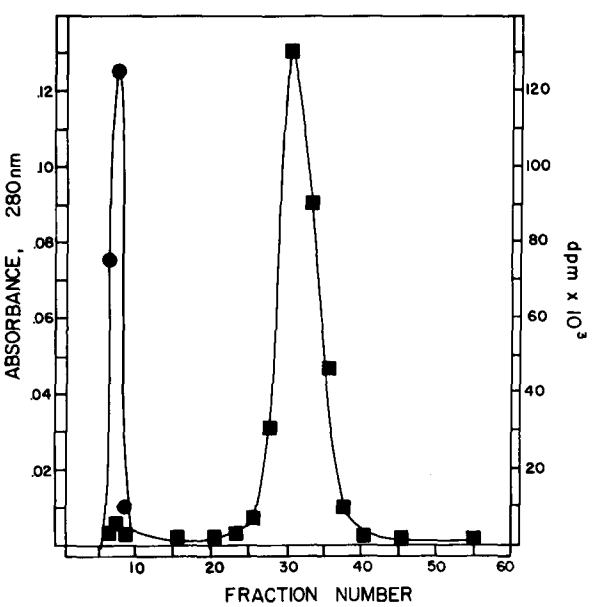

Fig. 4. Elution profile of Bio-Gel P-6 chromatography of 2,4-pentanedione-modified dihydrofolate reductase after incubation with a 2-fold molar excess of [^{3}H]amethopterin. 280 nm, ●; dpm, ■.

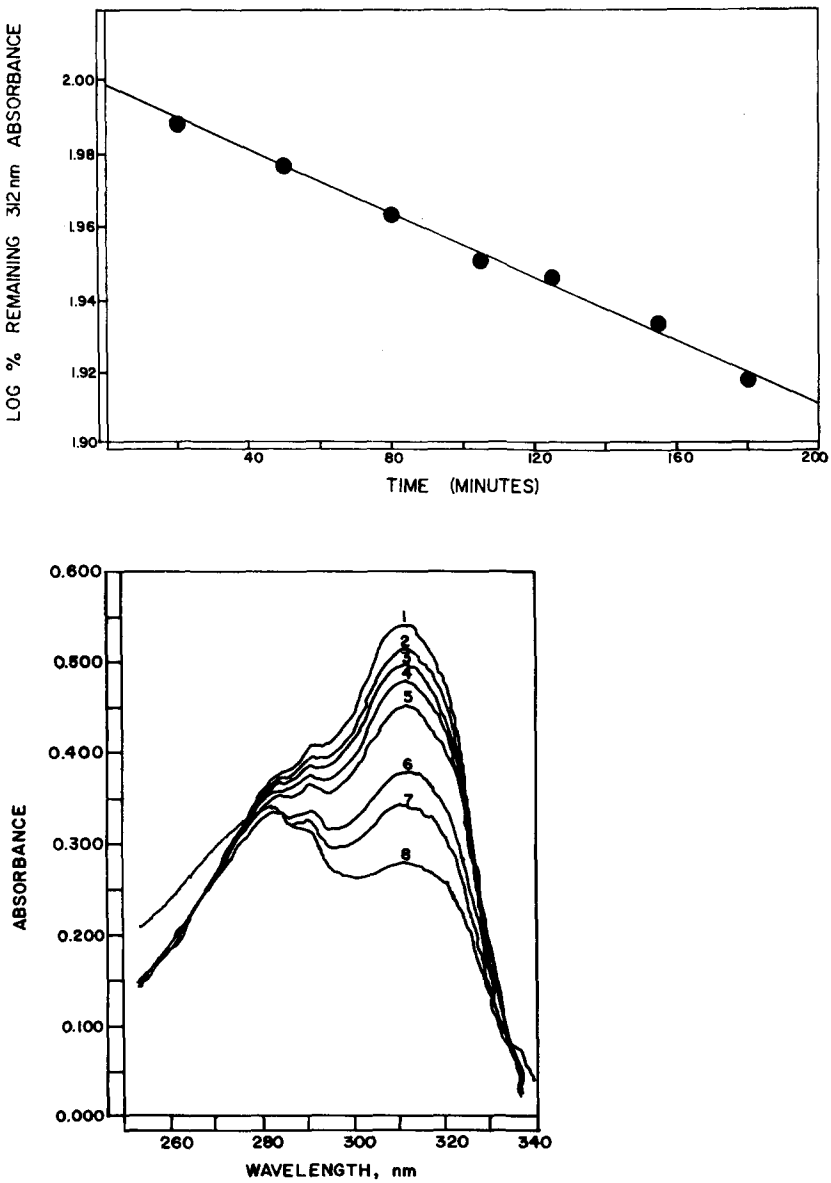

Fig. 5. (A) Time course for the loss of enamine of 2,4-pentanedione-modified dihydrofolate reductase incubated at room temperature. (B) Actual spectral scans of enamine breakdown as a function of time of incubation of 2,4-pentanedione-modified enzyme; curve 1, 0 min; 2, 50 min; 3, 80 min; 4, 105 min; 5, 180 min; 6, 423 min; 4, 578 min, and 8, 1005 min.

Dihydrofolate and amethopterin also form binary complexes with native dihydrofolate reductase which are stable to Bio-Gel P-6 chromatography (Bednarek, J.M. and Dunlap, R.B., unpublished data). When 2,4-pentanedione-modified dihydrofolate reductase was treated with either [^{3}H]dihydrofolate or [^{3}H]amethopterin and chromatographed on Bio-Gel P-6, no radioactivity was found to be associated with the enzyme as shown in Figs. 3 and 4, respectively.

TABLE II

REACTIVATION OF 2,4-PENTANEDIONE-MODIFIED DIHYDROFOLATE REDUCTASE AFTER GEL FILTRATION

V/V_c (×100%) *	V/V_c (×100%) after gel filtration (h) **			
	0	1	2	3
16	16	16	16	16
35	58	72	72	—
48	64	86	91	91
80	100	100	100	100

* Dihydrofolate reductase (0.1 mg/ml) was modified by 0.2 M 2,4-pentanedione. Activity remaining is expressed as the ratio V/V_c (×100%) where V is the activity of 2,4-pentanedione-modified enzyme and V_c is the activity of an unreacted control enzyme.

** 2,4-Pentanedione-modified enzyme was subjected to gel filtration on Sephadex G-10 in 0.1 M potassium phosphate, 0.1 M KCl (pH 6.0). Enzyme was incubated at 30°C and periodically assayed for activity.

Reactivation studies and stability of the enamine

2,4-Pentanedione-modified enzyme, inactivated to 20% of its native activity, was treated with hydroxylamine, methoxyamine, pyridine-2-aldoxime methiodide, 2-mercaptoethanol, dithiothreitol or bisulfite, but no reactivation of the enzyme was observed. Treatment of modified enzyme with hydroxylamine followed either by incubation at 25, 33 or 41°C or by extensive dialysis at 4°C did not result in reactivation of the enzyme. Although hydroxylamine was ineffective in reversing inactivation of the enzyme, this nucleophile did cause an immediate and substantial decrease in the magnitude of the 312 nm peak which was followed by an increase in the turbidity of the sample. Reactivation was achieved by passing inactivated enzyme down a Sephadex G-10 column which had been equilibrated in 0.1 M potassium phosphate, 0.1 M KCl, pH 6.0, and allowing the fractions containing enzyme to incubate at 30°C. However, the degree of reactivation was dependent on the extent to which the enzyme was inactivated (Table II).

A sample of modified enzyme, which had been inactivated to 19% of its original activity and chromatographed on a Sephadex G-10 column to remove excess 2,4-pentanedione, was monitored spectrophotometrically from 340 to 250 nm for 18 h at 25°C and pH 7.0. The rate of decrease in absorbance at 312 nm as a function of time followed pseudo first-order kinetics and resulted in an apparent first-order rate constant of $4.38 \cdot 10^{-4}$ min (Fig. 5). However, after loss of 50% of the absorbance due to the enamine, no enzymic activity was regained.

Discussion

Recent sequence analysis on dihydrofolate reductases from *L. casei* [3,14], *Streptococcus faecium* [15], and *Escherichia coli* [16], shows identities of 29% and 34% between the *L. casei* enzyme and the reductases of *E. coli* and *S. faecium*, respectively. If minimum single-base changes are considered, the

L. casei shows identities of 73% and 64% with the respective *S. faecium* and *E. coli* enzymes [3]. These studies, coupled with the X-ray crystallographic determination of the *E. coli* [17] and *L. casei* [18] enzymes place lysyl groups at or near both the coenzyme and folate binding regions. The fact that the substrates involved in the dihydrofolate reductase reaction are anionic in nature led us to investigate the essentiality of cationic residues in the active site region of the enzyme. Arginyl residues have been implicated as essential residues in this enzyme both by chemical modification [5] and by NMR [19—21]. Vehar et al. [6] have implicated a lysyl residue at or near the NADPH binding region of this enzyme employing dansyl chloride as a modifying reagent. In the present study we have chosen 2,4-pentanedione as a selective lysine-modifying agent since it avoids some of the problems that may be inherent in the use of dansyl chloride such as tyrosine modification and steric hindrance. Indeed, Gilbert and O'Leary [7,8] and Hayman and Colman [9] have shown that, at the proper pH, 2,4-pentanedione is highly specific for lysine residues in their studies with lysozyme, aspartate aminotransferase and isocitrate dehydrogenase, respectively, although the possibility of N-terminal modification does exist [8].

Our results indicate that chemical modification of dihydrofolate reductase with 2,4-pentanedione specifically inactivates the enzyme with the subsequent modification of three residues of a total of nine lysines and one N-terminus. The fact that either NADPH or dihydrofolate reductase provides excellent protection against 2,4-pentanedione inactivation can be interpreted to position the critical lysyl residues at or near the coenzyme and/or folate binding regions. This is in accord with the protection afforded the enzyme by NADPH in the presence of dansyl chloride as observed by Vehar et al. [6]. It could be postulated that the critical residue(s) is involved in binding to the 2′-phosphate on the adenosine ring of NADPH and/or to the negatively charged glutamate function on dihydrofolate. Additional evidence for placing this residue(s) at these regions is indicated by the failure of either NADPH, dihydrofolate or amethopterin to bind to inactivated dihydrofolate reductase.

The absorption spectrum of 2,4-pentanedione-modified dihydrofolate reductase is essentially identical to that reported for 2,4-pentanedione-modified lysozyme [7], for modified isocitrate dihydrogenase [9] and for modified aspartate aminotransferase [8]. Spectrophotometric studies of the breakdown of the enamine as a result of incubation of modified dihydrofolate reductase at room temperature for extended periods of time were monitored at 312 nm and found to be similar to that reported by Hayman and Colman [9], although the rate of enamine breakdown was ten-fold slower in the present study. Although treatment of pentanedione-modified isocitrate dehydrogenase [9] and lysozyme [7] with hydroxylamine or incubation at acidic pH was reported to result in substantial regeneration of activity, we have not found this to be the case with dihydrofolate reductase. Treatment of the 2,4-pentanedione-modified reductase with 0.1 M hydroxylamine (pH 7.0) caused a rapid decrease in the absorbance at 312 nm due to enamine formation, but did not result in an increase of enzymatic activity. This result suggests the possibility of some mechanism of covalent attachment of the reagent to the enzyme following treatment with hydroxylamine. In contrast, native dihydrofolate reductase is unaffected by treatment with hydroxylamine. Treatment of modified enzyme

with other nucleophiles such as methoxyamine or pyridine-2-aldoxime methiodide were likewise unsuccessful in reactivating the modified enzyme.

Hayman and Colman [9] used radiolabeled 2,4-pentanedione to a great advantage in their studies with isocitrate dihydrogenase. Obviously, with labeled reagent one could easily ascertain whether the label was still associated with the inactivated enzyme following treatment with hydroxylamine. Furthermore, labeled reagent would greatly help in determining the actual number of critical residues for pyridine nucleotide binding or for folate binding by treating enzyme in the presence of NADPH or amethopterin, respectively, with labeled reagent. Such quantitation has not been possible in our laboratory using unlabeled reagent since the absorption of the ligand bound to the enzyme interferes with the 312 nm absorbance. We intend to carry out these and other studies with labeled reagent at a future date.

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