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The Effect of Histidine Modification on the Activity of Dihydrofolate Reductase from a Methotrexate-Resistant Strain of *Escherichia coli* B†

Norma J. Greenfield

ABSTRACT: Dihydrofolate reductase from *Escherichia coli* strain MB 1428 contains five histidines. The enzyme has been reacted with ethoxyformic anhydride (EFA) at pH 7.2 and the treated enzyme has been assayed at both pH 7.2 and pH 4.7. The treatment with EFA rapidly ethoxyformylates two histidines; the rest react more slowly. At pH 7.2 there is a rapid 50–60% loss of enzymatic activity corresponding to the modification of the rapidly reacting histidines. When the enzyme modified at pH 7.2 is assayed at pH 4.7, however, there is a loss of 90% of the activity in the same time period. In the presence of dihydrofolate, methotrexate, or folic acid, one rapidly reacting histidine is protected from reaction with EFA at pH 7.2. There is no protection against the loss of activity at pH 7.2, but the folates protect against approximately half the activity loss at pH 4.7. The enzyme from *E. coli* MB 1428 has two nonequivalent NADPH binding sites. The binding of NADPH at the stronger of the two sites also protects one of the rapidly reacting histidines from ethoxyformylation at pH 7.2 and protects almost fully against the loss of activity at pH 7.2 and against approximately half of the activity loss at pH 4.7. When the dihydrofolate reductase is protected by both methotrexate and NADPH approximately 2 molar equiv of histidine are protected from rapid reaction with EFA suggesting that the histidines protected by the folates and NADPH are different. Fluorescence and circular dichroism experiments suggest that the modified enzyme exhibits no loss of binding capacity for NADPH, dihydrofolate, and methotrexate. The K_m's for dihydrofolate and NADPH are unchanged at pH 7.2 when compared to those of the native enzyme, but when the enzyme is modified at pH 7.2, but assayed at pH 4.7 the K_m for NADPH increases relative to that of the native enzyme. The pH–activity profile of the modified enzyme changes upon histidine modification; the pH optimum remains the same.

N-Bromosuccinimide oxidation of dihydrofolate reductase from a methotrexate (amethopterin)-resistant strain of *Escherichia coli* (strain MB 1428) has suggested histidine is involved in the active site (Williams, 1972, 1974). When dihydrofolate reductase is titrated with *N*-bromosuccinimide (NBS[1]) there is an initial loss of 40–50% of the enzymatic activity with no tryptophan oxidation. Amino acid analysis indicates that the initial loss of activity may correspond to the oxidation of one histidine. The partially modified protein, however, shows no loss of capacity to bind reduced nicotinamide adenine dinucleotide phosphate (NADPH), the cofactor involved in dihydrofolate reduction, or methotrexate, a potent inhibitor of the enzyme. Moreover, the modified protein has fluorescence and circular dichroism properties identical with the native enzyme (Williams, 1972, 1974). Further additions of NBS result in the modification of tryptophan residues, and partial loss of ability to bind both NADPH and methotrexate.

Efforts to determine whether substrates and cofactors protect against the initial modification by NBS are complicated because both dihydrofolate and NADPH interact with NBS. The reaction of both of these produces spectral changes in the ultraviolet region used to determine the extent of tryptophan modification, thus it is difficult to perform protection studies.

In order to explore more fully the role that histidine plays in the activity of dihydrofolate reductase it has been necessary to extend the study to a "histidine specific" reagent.

Modification of proteins with ethoxyformic anhydride (EFA) at acidic pH has been shown to be relatively specific

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[1] Abbreviations used are: NBS, *N*-bromosuccinimide; EFA, ethoxyformic anhydride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); N_2phF, fluorodinitrobenzene.

for histidine. The reaction with histidine can be easily quantitated by following the extent of reaction spectrophotometrically using a molar extinction coefficient of 3.2×10^3 at 242 nm (Ovadi *et al.*, 1967a). Mulrad *et al.* (1967) have shown that ethoxyformic anhydride can ethoxyformylate histidine, arginine, tyrosine, and sulfhydryls. However, at pH 6.0 the reagent seems specific for histidine (Ovadi *et al.*, 1967b) and sulfhydryl groups are not modified (Pradel and Kassab, 1968). Recently many other groups have used the reagent with good specificity for histidine at pH 6 (Setlow and Mansour, 1970; Holbrook and Ingram, 1973; Elodi, 1972). Melchior and Fahrney (1970), on the other hand, have shown that even at low pH (4.0) amino groups of proteins may also react with the reagent and recently Wells (1973) has found the EFA reacts with 1 molar equiv of lysine in phospholipase A and protects the lysine from fluorodinitrobenzene derivatization. Moreover, Burstein *et al.* (1974) have shown that when thermolysin is treated with EFA at pH 7.2 tyrosine residues are modified as monitored by a decrease in absorption at 278 nm, and Rosen and Fedorcsak (1966) have cited spectra changes at 280 and 240 as possible evidence of tryptophan modification of bovine serum albumin at pH 7 as well. The reaction with amino groups, however, is not reversed by treatment with hydroxylamine and thus can easily be distinguished from modification of histidine. Moreover, Burstein *et al.* (1974) have shown that mild treatment with hydroxylamine does not reverse tyrosine modification in their study. Since the addition of ethoxyformic anhydride to folates and NADPH gives negligible spectral changes at 242 nm the reagent seems ideal to study the effect of histidine modification on the activity of dihydrofolate reductase.

This work describes the reaction of dihydrofolate reductase with ethoxyformic anhydride, the effect of protection of the enzyme by substrates, inhibitors, and cofactors, and the properties of the modified protein. Since ethoxyformic anhydride is more specific for histidine at low pH, preliminary experiments were done at pH 6.0 as described by Ovadi *et al.* (1967), Setlow and Mansour (1970), and Pradel and Kassab (1968). However, the enzyme is less stable at pH 6.0 and tends to precipitate. Moreover, previous characterizations have been done in this laboratory at pH 7.2 (Greenfield *et al.*, 1972; Williams *et al.*, 1973a), thus subsequent work was performed in Tris-HCl at pH 7.2.

Experimental Section

Materials. Dihydrofolate reductase from *Escherichia coli*, strain MB 1428, was purified as described by Poe *et al.* (1973) as modified by Williams *et al.* (1973a,b).

Ethoxyformic anhydride (EFA) (diethyl pyrocarbonate, diethyloxy diformate) was obtained from Eastman Organic Chemicals. Folic acid dihydrate was purchased from Cyclo Biochemical Corp., methotrexate from Nutritional Biochemicals. NADPH was purchased from P-L Laboratories, Milwaukee. Tris(hydroxymethyl)aminomethane (Tris) and urea were ultrapure enzyme grade from Schwarz/Mann. Dihydrofolate was prepared from folic acid by the dithionite method of Futterman (1957) as modified by Blakley (1960) and stored at −20° in 5 mM HCl and 50 mM 2-mercaptoethanol.

p-Hydroxymercuribenzoate was obtained from Sigma. All other chemicals were reagent grade.

Standardization of Reagent. The concentrations of NADPH, methotrexate, folate, and dihydrofolate were determined spectrophotometrically using a molar extinction of $\epsilon_{340\,nm}$ 6220 for NADPH (Kornberg and Horecker, 1953), $\epsilon_{258\,nm}$ 23,250 and $\epsilon_{302.5\,nm}$ 22,100 for methotrexate at pH 13 (Seeger *et al.*, 1949); $\epsilon_{282\,nm}$ 27,000 for folate (Dawson *et al.*, 1969); and $\epsilon_{282\,nm}$ 28,000 for dihydrofolate (Dawson *et al.*, 1969). The concentration of dihydrofolate reductase was estimated using $\epsilon_{282\,nm}$ 40,000 (Greenfield *et al.*, 1972) or by direct fluorescence titration of the enzyme with methotrexate (Williams *et al.*, 1973a,b).

Spectroscopy. Circular dichroism measurements were done as described in Greenfield *et al.* (1972). Fluorescence measurements were performed as described by Williams *et al.* (1973a,b). Absorption spectra were recorded on a Cary 15 spectrophotometer.

Dissociation of Enzyme–Ligand Complexes. Dissociation constants were estimated by the method of Kurganov *et al.* (1972) as described by Greenfield *et al.* (1972) for one binding site. For multiple binding sites the method of Williams *et al.* (1973b) was used.

Enzyme assays were performed as described by Poe *et al.* (1972). Assays were performed either in 0.05 M Tris (pH 7.2) or 0.05 M acetate (pH 4.7). The standard assay buffer contained approximately 100 μM NADPH and 80 μM dihydrofolate. The reaction was initiated with enzyme.

The effect of pH on the enzymatic rate was measured as above. However, the measurements were performed in 0.01 M buffers containing 0.1 M NaCl. The buffers used were sodium acetate, sodium phosphate, and Tris-HCl.

Measurements of the Michaelis constants for the enzyme were performed in 0.05 M Tris-HCl (pH 7.2) and 0.05 M sodium acetate (pH 4.7). The K_m of the enzyme and enzyme modified with ethoxyformic anhydride for NADPH was measured at saturating concentrations of dihydrofolate of approximately 80 μM. The K_m for dihydrofolate of the native and modified enzyme were performed at a saturating NADPH concentration of 100 μM.

Ethoxyformylation. Ethoxyformylations were performed in 0.1 M phosphate buffer (pH 6.0); 0.05 M Tris–0.05 M NaCl (pH 7.2); and 0.05 M Tris–0.3 M NaCl (pH 7.2). Enzyme concentrations ranged from 5 to 20 μM. Directly before use a 1 or 2.5% solution of ethoxyformic anhydride in cold absolute ethanol was prepared. The concentrations of the ethanolic solutions were determined as described by Setlow and Mansour (1970); 1 ml of enzyme solution was treated with 10 μl of the reagent solutions. The reaction mixture was incubated for 15 min at 25°. The extent of ethoxyformylation of histidine was determined by monitoring the increase of absorbance at 242 nm, and was quantitated using the extinction coefficient of 3200 per mole of ethoxyformyl histidine formed (Ovadi *et al.*, 1967a). In parallel experiments the loss of activity was followed as a function of time.

Reversal of Ethoxyformylation of Histidine by Hydroxylamine. 0.5 M Hydroxylamine solutions at pH 7.0 in water were prepared; 100-μl aliquots of ethoxyformylated dihydrofolate reductase were mixed with equal volumes of hydroxylamine and the mixtures were incubated at 25° for 5 min and the enzyme was assayed. Controls using unmodified enzyme were run simultaneously.

Determination of Tryptophan. The total tryptophan content before and after ethoxyformylation was determined by titration of the enzyme with *N*-bromosuccinimide in 6 M guanidine-HCl as described by Williams (1974) using the method of Patchornik *et al.* (1958).

Determination and Masking of Reactive Sulfhydryl Groups. 5,5′-Dithiobis(2-nitrobenzoic acid) (Nbs_2), a re-

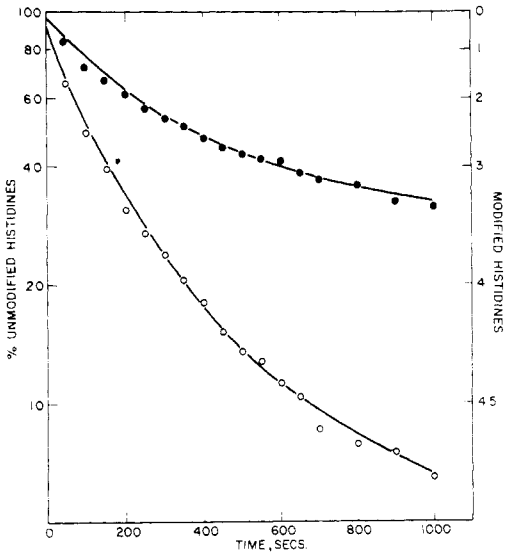

FIGURE 1: The modification of histidine residues of dihydrofolate reductase as a function of ethoxyformic anhydride concentration. (●) EFA, 0.01%, 250 μM; enzyme, 19 μM; ratio, 13:1. (○) EFA, 0.025%, 630 μM; enzyme, 19 μM; ratio, 43:1. Buffer is 0.05 M Tris-HCl–0.05 M NaCl.

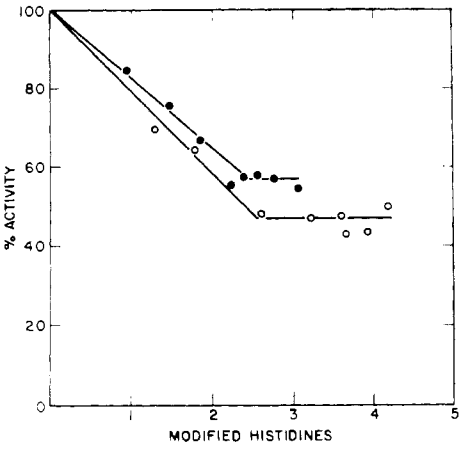

FIGURE 2: The correlation of the loss of activity of dihydrofolate reductase with histidine modification. (●) EFA, 0.01%, 250 μM; enzyme, 19 μM; ratio, 13:1. (○) EFA, 0.025%, 630 μM; enzyme, 19 μM; ratio, 43:1. Buffer is 0.05 M Tris-HCl–0.05 M NaCl.

agent described by Ellman (1959, 1961), was used to determine the number of reactive sulfhydryl groups before and after modification with EFA. The procedure of Williams (1974) was used; 50 μl of 4×10^{-3} M Nbs_2 was added to 0.8 ml of ethoxyformylated enzyme dissolved in 0.05 M Tris-HCl–0.3 M NaCl (pH 7.2) and to ethoxyformylated enzyme dissolved in 0.05 M Tris-HCl–0.3 M NaCl–6 M guanidine-HCl–10^{-3} M EDTA. The number of reactive thiol groups were calculated from the change of absorbance at 412 nm using glutathione as a Nbs_2 standard under the same conditions.

Dihydrofolate reductase has two cysteines (Bennett, 1974). In the absence of denaturing agents only one of these is modified by Nbs_2 or *p*-hydroxymercuribenzoate (Williams and Hoogsteen, 1974). Nbs_2 was used to mask the reactive sulfhydryl by treating enzyme with approximately a 20 molar excess of Nbs_2. The extent of reaction was monitored utilizing the change in absorbance at 412 nm. As soon as the reaction was over, the enzyme was stripped of excess Nbs_2 on a Sephadex G-25M column equilibrated with 0.05 M Tris-HCl–0.3 M NaCl (pH 7.2) and the peak fraction was immediately treated with EFA.

Determination of Lysine. The number of lysines modified by ethoxyformic anhydride was determined as described by Wells (1973); 3 ml of approximately 3 μM enzyme in 0.05 M Tris-HCl–0.05 M NaCl (pH 7.2) was treated with 30 μl of 1% ethoxyformic anhydride for 15 min. The extent of histidine modification was monitored by following the OD change at 242 nm. The enzyme was cooled in ice and the activity was monitored. The solution was diluted to 10 ml with distilled water and dialyzed against distilled water at 4° overnight. The enzyme was lyophilized to dryness and dissolved in 0.25 ml of 1% triethylamine; 0.5 ml of 5% fluorodinitrobenzene in 95% ethanol was added, and the solutions were allowed to sit for 3 hr at 23° in the dark; 0.5 ml of distilled water was added and the solutions were extracted until the ether phase was colorless. The aqueous layer was heated on a steam bath to remove traces of ether and this layer was lyophilized. A control sample without ethoxyformic anhydride was also prepared and the samples were acid hydrolyzed and amino acid analyses were performed. The number of lysines which reacted with EFA was determined by the number of lysines detected in the amino acid analyses; *i.e.*, they were the lysines which were protected from reaction with fluorodinitrobenzene.

Amino Acid Analysis. Acid and alkaline hydrolysis were performed as described by Poe *et al.* (1972).

Results

Modification of Dihydrofolate Reductase with Ethoxyformic Anhydride, pH 6.0. Preliminary modification experiments were done at pH 6.0 in 0.1 M phosphate buffer. In a typical experiment 1 ml of 15 μM dihydrofolate reductase was treated with 10 μl of 1% ethoxyformic anhydride dissolved in absolute ethanol and the mixture was incubated at 25°. The extent of reaction with histidine was followed by monitoring the increase in absorption due to the formation of the ethoxyformylated histidine. Within 15 min, approximately three histidines reacted resulting in a 50% loss of activity. Further incubation caused no further histidine modification or activity loss. The activity loss was reversed by treating the enzyme for 5 min with 0.25 M hydroxylamine at pH 7.0. This suggested that the activity loss was due to histidine modification.

pH 7.2. At pH 7.2 the extent of histidine modification was studied as a function of ethoxyformic anhydride concentration. Figure 1 shows the log of the number of unreacted histidine residues as a percentage of the total number of histidines present in the protein plotted against the time of reaction. The percentage of slowly reacting groups was determined by extrapolating the slope of the latter portion of the curve (in this one case the last 500 sec) back to the origin. The remaining groups were defined as "rapidly" reacting (Cohen, 1970). At a 13-fold excess of ethoxyformic anhydride to enzyme approximately 3.5 histidines were modified within 1000 sec. At a 43-fold excess of approximately 4.7 of the five histidines of dihydrofolate reductase were modified within this time. The plots suggest that two histidines react very fast, one moderately fast, one slowly, and the last very slowly. At both concentrations of EFA approximately 50% of the activity was lost when the enzyme was assayed at pH 7.2. Figure 2 shows the loss of activity at pH 7.2 correlated with histidine modification.

Since the possibility of side reactions are increased at pH

TABLE I: Loss of Activity of Dihydrofolate Reductase at pH 7.2 upon Ethoxyformylation at pH 7.2 and Reversal by Hydroxylamine.[a]

Sample	ΔA_{340} min^{-1} ml^{-1}	% Native Rate	% Control
Untreated enzyme	13,000 ± 2500	100	
Enzyme + EFA	6,000 ± 2500	46	
Enzyme + hydroxylamine	12,100 ± 1500	93	100
Enzyme + EFA + hydroxylamine	11,500 ± 1000	88	95

[a] Samples were treated for 15 min with a 0.01% solution of ethoxyformic anhydride (EFA) so that 3.8 of the 5 histidines in the molecule were ethoxyformylated. In the reversal experiments equivolumes of 0.5 M hydroxylamine (pH 7.0) and ethoxyformylated enzyme were mixed and incubated 5.0 min.

7.2 several tests were performed to assure that the loss of activity is due to histidine modification.

The first test was reversal of the activity loss by treatment with hydroxylamine: a typical result is shown in Table I. In this case a sample of enzyme in 0.05 M Tris–0.3 M NaCl (pH 7.2) was treated with 0.01% ethoxyformic anhydride. After 15 min 3.8 of the 5 histidines reacted and the enzyme activity dropped to 46% of the native activity. When treated with an equivolume of 0.5 M hydroxylamine for 5 min the activity returned to 88% of the native. Unfortunately, hydroxylamine treatment alone causes a 7% loss of activity in the control sample, thus the EFA treated enzyme regained 95% activity relative to the control. Other samples showed up to 100% reversal relative to controls.

Dihydrofolate reductase from *E. coli* B, MB 1428 has five tryptophans. There were no differences in tryptophan content between the native and modified enzyme when measured by the method of Patchornik *et al.* (1958). Moreover, there were no optical spectral changes above 270 nm, hence no tyrosines were modified.

The reductase from *E. coli* has two sulfhydryl groups (Poe *et al.*, 1972; Bennett, 1974). Only one is exposed in the native enzyme (Williams and Hoogsteen, 1974). When the enzyme was modified with EFA under conditions where four of the five histidines reacted and then treated with Nbs_2 using "native" conditions in 0.05 M Tris-HCl–0.3 M NaCl, 0.8 cysteine residue reacted in the modified enzyme while 1.0 group reacted in the untreated control. When the unmodified and ethoxyformylated enzyme were titrated with Nbs_2 in 0.05 M Tris-HCl–0.3 M NaCl–6 M guanidine-HCl–10^{-3} M EDTA as described by Williams (1974) 2.0 sulfhydryl groups reacted in the unmodified enzyme but only 1.8 reacted in the enzyme treated with EFA.

Thus ethoxyformic anhydride apparently modifies 20% of the readily accessible sulfhydryl group of dihydrofolate reductase. The modification of the accessible sulfhydryl group, however, with either Nbs_2 or *p*-hydroxymercuric benzoate causes no loss of activity (Williams and Hoogsteen, 1974). In order to assure that none of the activity loss from ethoxyformylation was caused by the 20% sulfhydryl modification by EFA, the reactive sulfhydryl was first modified with Nbs_2, and then the enzyme was modified with

TABLE II: Amino Acid Analysis of Dihydrofolate Reductase from *E. coli* B (Strain MB 1428) Treated with Ethoxyformic Anhydride.

Residue	Expected from Sequence[a]	Control[b]	Ethoxyformylated Enzyme
Lys	7	7.6	4.2
His	5	4.8	6.0
Arg	9	8.3	8.2
Asp	18	18.2	18.3
Thr	6	6.2	5.3
Ser	8	8.7	8.7
Glu	15	16.1	14.4
Pro	10	9.6	9.1
Gly	10	10.4	9.1
Ala	13	12.6	12.5
Val	11	10.4	9.5
Met	5	3.6	4.2
Ile	11	9.3	9.7
Leu	11	11.0	10.6
Tyr	4	4.0	4.5
Phe	6	6.0	6.6

[a] Bennett (1974). [b] Average of three experiments.

EFA. Both the native enzyme and the enzyme protected by Nbs_2 showed identical modification of histidine residues and identical activity loss.

Dihydrofolate reductase has seven lysines. An amino acid analysis of the enzyme treated with EFA was low in lysines, but there were no other significant changes. (The amino acid analysis is shown in Table II.) The modification of lysine was confirmed by the fluorodinitrobenzene procedure of Wells (1974).

When dihydrofolate reductase was treated with EFA so that four of the five histidines reacted with EFA, and subsequently reacted with N_2phF, 4.2 lysines did not react with N_2phF. Only 1.2 lysines did not react in the enzyme which was not ethoxyformylated. Thus three lysines apparently react with EFA. The ethoxyformylation of the lysine residues are not responsible for the loss of activity, however, since hydroxylamine treatment does not reverse the ethoxyformylation of lysine.

Circular Dichroism (CD) Measurements. There were no differences between the CD patterns of the native enzyme and the enzyme modified with ethoxyformic anhydride. CD was also used to monitor the binding of dihydrofolate and methotrexate to the enzyme, and to measure the binding of NADPH to the enzyme complex as described in Greenfield *et al.* (1972). In each case the results were identical for the native and modified enzyme.

Fluorescence Measurements. When fluorescence was used to examine the enzyme modified with ethoxyformic anhydride there were slight differences compared to the native enzyme. When excited at 290 nm, the fluorescence emission at 350 of the modified enzyme is 88.5% of that of the native enzyme. When the native and modified enzymes were titrated with methotrexate in 0.05 M Tris–0.3 M NaCl the dissociation constants of the native and modified enzymes were almost identical. They were $1.3 \pm 0.5 \times 10^{-8}$ and $1.6 \pm 0.9 \times 10^{-8}$ M, respectively. These values are within close agreement with the values reported by Wil-

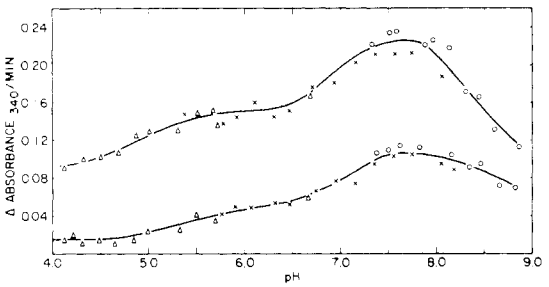

FIGURE 3: The pH-activity profile of dihydrofolate reductase and dihydrofolate reductase modified with ethoxyformic anhydride at pH 7.2 in 0.01 M buffers plus 0.1 M NaCl. (Δ) acetate; (X) phosphate; (O) Tris-HCl; top curve, native enzyme; lower curve, modified enzyme.

liams *et al.* (1973a) of $2.7 \pm 0.6 \times 10^{-8}$ M. The molar extinction coefficients determined from these titrations were also identical. However, methotrexate quenched 82% of the fluorescence of the native enzyme and only quenched 70% of the fluorescence of the modified enzyme.

The binding of NADPH to the enzyme was studied using the enhancement of NADPH fluorescence upon binding to the enzyme as described by Williams *et al.* (1973b). The enzyme modified with EFA had the same dissociation constants for NADPH as the native enzyme in buffers of both 0.10 and 0.35 ionic strength. There were slight changes in the fluorescence parameters, however, as the enhancement of fluorescence of NADPH when complexed to the enzyme was slightly lower in the modified protein.

The pH Dependence of the Activity of Dihydrofolate Reductase and Dihydrofolate Reductase Modified with Ethoxyformic Anhydride. Figure 3 shows the effect of pH on the maximum activity of the modified and unmodified enzyme. The studies were performed in 0.1 M NaCl in 0.01 M acetate, phosphate, and Tris-HCl buffers. The activity of dihydrofolate reductase is strongly ionic strength dependent (Poe *et al.*, 1972); thus the concentration of buffer was kept low in order to minimize the effect of changes of ionic strength as a function of pH. The unmodified enzyme has a pH optima at pH 7.3–8.0 with a shoulder in the activity at approximately pH 5.5. Below pH 5 and above pH 8 the enzyme activity is diminished. Thus, it would appear that there are at least three ionizable groups on either the substrates or the enzyme that appear to affect activity. Modification with ethoxyformic anhydride so that three of the five histidines reacted did not change the pH optimum of 7.3–8.0, but greatly decreased the activity at pH 5.0. At pH 7 and above the enzyme only lost 50% of its activity, but below pH 5 the enzyme lost 90% of its activity.

K_m Values of Dihydrofolate and NADPH at pH 7.2 and 4.7. The K_m's of the modified and unmodified enzyme are shown in Table III. The K_m for NADPH is sevenfold higher in the modified enzyme at pH 4.7. All other K_m values are within experimental error.

TABLE III: The Kinetic Constants of Dihydrofolate Reductase Modified with Ethoxyformic Anhydride.

pH	Ligand	K_m, μM	
		Unmodified	Modified
7.2	Dihydrofolate	1.2 ± 0.5	2.7 ± 1.3
	NADPH	3.9 ± 2.0	2.1 ± 0.8
4.7	Dihydrofolate	2.5 ± 1.4	3.7 ± 1.3
	NADPH	0.2 ± 0.1	1.4 ± 0.5

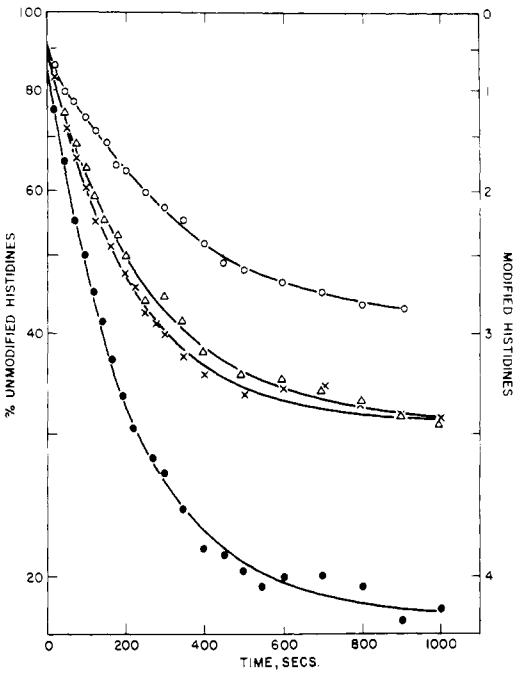

FIGURE 4: The protection of dihydrofolate reductase from ethoxyformic anhydride modification by NADPH and methotrexate. Enzyme, 12 μM in 0.05 M Tris-HCl–0.3 M NaCl (pH 7.2). (●) enzyme alone; (Δ) enzyme + 4:1 NADPH; (X) enzyme + 2:1 methotrexate; (O) enzyme + 2:1 methotrexate + 4:1 NADPH; EFA, 0.025%; EFA:ENZ, 50:1.

Protection against Ethoxyformylation by Substrates, Cofactors, and Inhibitors. Since the extent of ethoxyformylation is a function of the enzyme and anhydride concentration, all protection experiments were run in parallel with a control under identical conditions. The results are reported relative to the unprotected control. The incubation of dihydrofolate with EFA produced no spectral changes during the time course of the experiments, however, the incubation of NADPH produced an approximate 3% increase in magnitude of the bands at both 340 and 260 nm. There is also the possibility that dihydrofolate reacts with EFA without producing spectral changes. The protection experiments were therefore run with several different concentrations of EFA to ensure that the protections noted were not due to consumption of reagent by the substrates. Since the binding of NADPH and methotrexate are salt dependent, buffers containing either 0.05 or 0.3 M NaCl were used. Unless specifically stated the ionic strength of the solution had no effect on the experimental results.

pH 7.2: Folate Derivatives. Folate, dihydrofolate, and methotrexate each protect (*i.e.*, cause a rapidly reacting histidine to become slowly reacting) one rapidly reacting histidine from ethoxyformylation within the time course of the ethoxyformylation experiments of 1000 sec. However, when the enzyme is assayed to pH 7.2 neither folate nor dihydrofolate protect against any of the loss of activity. The protection of dihydrofolate reductase from ethoxyformylation by methotrexate is illustrated in Figure 4.

pH 7.2: NADPH. Dihydrofolate reductase has two strong binding sites for NADPH (Williams *et al.*, 1973b). At 0.1 ionic strength (0.05 M Tris-HCl–0.05 M NaCl at pH 7.2) the first binding site has considerably higher affinity for NADPH than the second (K_1 = 0.02 μM, K_2 = 0.6

μM). When a molar equivalent of NADPH is added to the enzyme under these conditions almost all of it binds at the higher affinity site. When exactly 1:1 NADPH was added to the enzyme and it was treated with ethoxyformic anhydride, one fast reacting histidine was protected from reaction with the reagent. Moreover, the NADPH protected against most of the activity loss. When an excess of NADPH was added to the enzyme to fill both sites, no further protection was noted.

At 0.35 ionic strength (0.05 M Tris-HCl-0.3 M NaCl (pH 7.2)) the binding affinities for NADPH by the enzyme are almost equivalent (K_1 = 0.2 μM, K_2 = 0.4 μM) (Williams *et al.*, 1973b). Thus, when a 1:1 molar equivalent of NADPH is added to the enzyme one would expect half the NADPH that binds to go into the first site and the other half to go into the second site. The binding sites are sufficiently strong that most of the NADPH added (>90%) is bound to the enzyme. When dihydrofolate reductase was treated with ethoxyformic anhydride in this buffer approximately 0.5–0.7 mol of histidine were protected from reaction. An excess of the cofactor protected exactly one histidine from reaction, and also protected against the activity loss at pH 7.2. The protection of the enzyme by an excess of NADPH is also shown in Figure 4.

The fact that NADPH protected against loss of activity, and dihydrofolate did not, when the enzyme was assayed at pH 7.2, suggested that the folates and cofactor might be protecting different histidines from reaction. Dihydrofolate reductase forms a complex with 1 molar equiv of methotrexate and 2 molar equiv of NADPH (Williams *et al.*, 1973b). When the inhibitor and cofactor are simultaneously added to the enzyme approximately two histidines are protected from rapid reaction as shown in Figure 4.

pH 4.7. When the enzyme was protected by either dihydrofolate or NADPH and reacted with ethoxyformic anhydride at pH 7.2, but assayed at pH 4.7, both the substrate and the cofactor protected against approximately half the loss of activity. When the activity of the hydroxylamine-treated enzyme was monitored at pH 4.7 it was found that only 80% of the native activity was regained after incubation for 5 min relative to the control. When the enzyme was protected by either dihydrofolate or NADPH, however, the enzyme regained 100% of the activity relative to a control. This suggests that at pH 4.7 most of the activity loss is indeed due to histidine modification, but that at the low pH the modified enzyme is less stable than the native and may lose some activity in the absence of protective substrates due to nonspecific denaturation. Alternately the 20% loss of activity that is not reversed by hydroxylamine treatment could be due to the modification of one of the lysine residues. The experiment is shown in Table IV. The results also suggest that the modification of the histidine that is protected by dihydrofolate does not affect the activity at neutral pH, but is responsible for loss of activity at pH 4.7.

TABLE IV: Loss of Activity at pH 4.7 of Dihydrofolate Reductase upon Ethoxyformylation at pH 7.2 and Reversal by Hydroxylamine.

Sample	ΔA_{340} min^{-1} ml^{-1}	% Native Rate	% Control
Untreated enzyme	5500	100	
Enzyme + EFA	870	16	
Enzyme protected with NAPH, + EFA	2060	37	
Enzyme protected with dihydrofolate, + EFA	1970	36	
Enzyme + hydroxylamine	5000	91	100
Enzyme + EFA + hydroxylamine	4000	73	80
Enzyme protected with NADPH, + EFA + hydroxylamine	5100	93	102
Enzyme protected with dihydrofolate, + EFA + hydroxylamine	5100	93	102

Discussion

Dihydrofolate reductase from the methotrexate resistant strain of *E. coli* B (MB 1428) contains five histidines.

When the enzyme is treated with EFA the extent of modification with time is concentration dependent and depending on conditions three to five of the histidines are modified. However, activity loss can be correlated with the modification of the first two "rapidly" reacting histidines.

The activity profile of dihydrofolate reductase is complex. There is an activity maximum at pH 7.2, but there is a pronounced shoulder of activity at pH 5.5. Several researchers have shown that dihydrofolate reductase from many different sources, *e.g.*, chicken liver, mammalian tissue, etc., have double pH optima (Mathews and Huennekens, 1963; Kaufman, 1964; Kaufman and Gardiner, 1966; Reyes and Huennekens, 1967). The effects of pH vary with ionic strength. It is possible the dihydrofolate reductase from *E. coli* B (MB 1428) also has two distinct enzymatic activity peaks which overlap and that the two peaks have different enzymatic mechanisms; or more likely, that the rate-determining step of the reductase reaction changes with pH.

At pH 7.2 all the activity loss upon ethoxyformylation can be correlated with the modification of a single histidine which is protected when NADPH binds at the tighter of its two binding sites. The protection may be direct, or NADPH binding may cause a conformational change which protects a histidine. The modification does not change the binding constant or Michaelis constant of NADPH for the enzyme. Since the modification does not cause a complete loss of enzyme activity it is possible that this histidine is not directly involved in the catalytic process of dihydrofolate reductase, but may somehow be involved in the regulation of enzyme activity.

When the enzyme is modified at pH 7.2 but assayed at pH 4.7, both NADPH and dihydrofolate protect partially against enzyme activity loss. Moreover, the Michaelis constant for NADPH increases at this pH.

Mulrad *et al.* (1967) and Melchior and Fahrney (1970) have shown that ethoxyformylation shifts the pK of histidine from approximately 7 to 4. Unfortunately, it is difficult to ascribe changes in the activity of dihydrofolate reductase to simple shifts in the ionization of histidine residues. When the reductase is treated such that three of the five histidines have reacted the modified and unmodified enzymes have the same pH optimum of 7.2–8.0 although the activity is decreased by 50% at these pH values. However, the shoulder at pH 5 is almost eliminated by ethoxyformylation (see Figure 3 for the pH profile). Since the pH

optimum near neutrality is unchanged by ethoxyformylation it is unlikely that a simple shift in the pK of a histidine residue is responsible for the activity loss at pH 7.2.

An interesting question is whether the modification by ethoxyformic anhydride has the same effect as the modification of a histidine by NBS. NBS modification of one histidine of dihydrofolate reductase also causes a 50% loss of activity when the activity is measured at pH 7.2 (Williams, 1972, 1974). The loss turned out to be coincidental, as the pH profile of the NBS modified enzyme is quite distinct from that of the enzyme treated by ethoxyformic anhydride. In the NBS treated enzyme, the pH optimum changes to lower pH (7.2 to 5.5) and there is only one activity peak. However, NADPH did protect dihydrofolate reductase from loss of activity, when the enzyme was treated with NBS, although the results are complicated because NADPH is oxidized by the reagent and tryptophan residues are also modified by NBS (Williams, 1974). It has been observed by Williams (personal communication) that if the enzyme is treated with NBS to cause a 40% loss of activity due to the destruction of histidine and then treated with the ethoxyformylating reagent, there is only a slight further loss of activity and this activity is only partially regained upon treatment with hydroxylamine. Furthermore, if one pretreats the enzyme with ethoxyformic anhydride and then treats with NBS, there is no initial loss of activity before tryptophan is oxidized. These results suggest that at least one of the histidines ethoxyformylated might be the same as the histidine which is modified by N-bromosuccinimide, but that the modifications are sufficiently different to have different effects.

The simplest explanation of the ethoxyformylation results is that there is a histidine at the tighter NADPH binding site which causes a loss of catalytic activity when modified. We are currently performing proton magnetic resonance studies of the enzyme, and studying the interactions of folates and pyridine nucleotides with the enzyme. The five histidines of dihydrofolate reductase are all resolved (Poe *et al.*, 1973) and it should be possible to gain greater detailed information on the role of histidine in the activity of the enzyme.

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