Protection of Microbial Dihydrofolate Reductase against Inactivation by Pronase

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SUMMARY

The dihydrofolate reductase of *Escherichia coli* is rapidly and irreversibly inactivated in the presence of the proteolytic enzyme Pronase. However, the reductase is almost completely protected when dihydrofolate, NADP, NADPH, or N^5-formyltetrahydrofolate are present at saturating concentrations. NAD, NADH, 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine, *p*-aminobenzoylglutamate, and glutamate were all ineffective in this regard. On the other hand, NAD did not accelerate the inactivation of *E. coli* dihydrofolate reductase by Pronase. Certain small-molecule, heterocyclic inhibitors of dihydrofolate reductase were also excellent protective agents when added at 30 times the concentration needed for 50% inhibition of the reductase. Surprisingly, at this same concentration certain other inhibitors with equal or better capacity for binding to the reductase failed to show any protective activity. The structural requirements for protection were explored, and some possible implications of the findings are discussed.

INTRODUCTION

Several reports now indicate that the binding of small molecules by proteins increases the resistance of the latter to hydrolysis by proteolytic enzymes. For example, Trayser and Colowick (1) have noted that the proteolytic digestion of hexokinase by trypsin is markedly diminished in the presence of glucose. Markus (2) has reported decreased proteolysis of serum albumin in the presence of methyl orange, and Grossberg *et al.* (3) have observed that a hapten–antibody complex is more resistant to proteolysis than is the antibody alone. The latter authors suggest that resistance to proteolysis may provide a highly sensitive tool for the detection of conformational changes accompanying the binding of small molecules to protein.

Recent experiments carried out in our laboratory with extracts of *E. coli B* (4) and by Hakala and Suolinna with cell-free preparations of Sarcoma 180 (5) provide evidence that the enzyme, dihydrofolate reductase, is protected against proteolytic degradation by its substrates, cofactor, and certain of its inhibitors. This paper expands and provides detailed information on our previous observations. Of particular importance is the finding that certain inhibitors known to bind tightly to bacterial dihydrofolate reductases are not capable of decreasing the rate of destruction of the reductase in the presence of the proteolytic enzyme Pronase (6).

METHODS AND MATERIALS

Protection experiments were carried out in the following manner: Partially purified preparations of *E. coli* dihydrofolate reductase prepared as described previously (7) were used in all experiments. These preparations were purified approximately 100-fold and were free of all pyridine nucleotide oxidases. Dihydrofolate reductase constituted about 1% of the total protein.

Aliquots of the purified reductase were incubated at 25° in the presence of 0.05 M

Tris buffer, pH 7.9, 0.1 M mercaptoethanol, 10–200 μg/ml of Pronase and the candidate protector in a total volume of 3.0 ml. When experiments were conducted involving the use of folic acid, dihydrofolic acid, reduced pyridine nucleotides, or methotrexate, the reaction systems were supplemented with sodium ascorbate (5 mg/ml) and incubated under N_2–CO_2 (95%–5%).

The inhibitors, when used, were added at a concentration 10–30 times that required to inhibit the *E. coli* dihydrofolate reductase by 50%. These concentrations were sufficiently high to allow the saturation of dihydrofolate reductase, yet dilution of 0.1 ml of the inhibited systems into the 3.0 ml reductase assay system (dilution 1:30) reduced the inhibition of the protected system to 50% or less and permitted measurement of changes in reductase activity during the course of the incubation. Control systems consisted of the complete system lacking either Pronase, the candidate protector or both.

As soon as the system containing Pronase but no protector was completely inactivated (usually about 1 hour), all the reaction systems were chilled in an ice bath, placed in dialysis sacs, and dialyzed overnight against ice-cold 0.05 M sodium acetate buffer, pH 5.5 with 1 mM EDTA. Under these conditions of low temperature and low pH, the activity of Pronase was almost entirely inhibited during the period when the inhibitor was diffusing from the tubing. The following morning the activity of all the systems was rechecked to be certain that the activity of the system containing the candidate protector and Pronase was equal to that of the system containing only dihydrofolate reductase.

At least three measurements were obtained on each system before inactivation of the control lacking a protective agent was complete. In this manner, it was possible to calculate accurately a percent inactivation by comparison of the rate of inactivation of the system with the candidate protector and Pronase to the rate of inactivation of the system with Pronase but without protector.

The assay of dihydrofolate reductase was based on the decrease in absorbancy at 340 mμ observed in the presence of dihydrofolate, NADPH, and enzyme (7). Dihydrofolate was prepared according to the method of Futterman (8) as modified by Blakley (9). Folic acid, dihydrofolic acid, methotrexate, calcium leucovorin, reduced and oxidized pyridine nucleotides, pABG, glutamic acid, and sodium ascorbate were all obtained from commercial sources. 2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine and all the dihydrofolate reductase inhibitors used in this study except methotrexate were synthesized previously in the Wellcome Research Laboratories. Preparations of 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine were purified free of oxidized pteridine prior to use by gradient elution from DEAE-cellulose between 0.005 M and 0.2 M Tris buffer, pH 7.2 with 1×10^{-3} M mercaptoethanol.

RESULTS

Figure 1 shows the results of an experiment which demonstrates the capacity of dihydrofolic acid to protect the dihydrofolate reductase of *E. coli* from inactivation by Pronase. The activity of the unprotected enzyme was rapidly lost in the presence of 150 μg/ml of Pronase. However, when dihydrofolate (10^{-4} M) was present, greater than 80% of the activity remained after 135 min. When the concentration of dihydrofolate was lowered to 10^{-6} M, reductase activity was lost at one-half the rate of the unprotected control system.

An interesting aspect of the protection effect is the complete inability of Pronase to inactivate dihydrofolate reductase in crude extracts of *E. coli*. Passage of crude extracts of *E. coli* over a Sephadex G-75 column yields fractions with peak absorbance at 260 mμ which, when added back to the partially purified reductase, protect that enzyme from inactivation by Pronase. These fractions appear to protect the reductase by inactivating Pronase, a conclusion based on the fact that aliquots of these fractions also diminish the activity of Pronase when casein is employed as a substrate for proteolysis. Preparations that

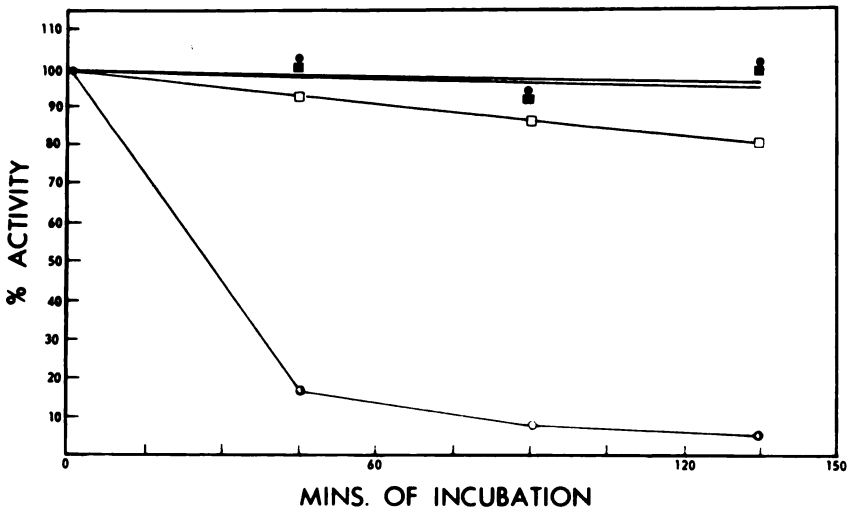


FIG. 1. *Kinetics of inactivation of Escherichia coli dihydrofolate reduction in the presence and absence of dihydrofolate*

Reaction systems contained *E. coli* dihydrofolate reductase (see Methods and Materials) and the following additions: ●——●, none; ■——■, dihydrofolate (10^{-4} M); □——□, dihydrofolate (10^{-4} M) + Pronase (150 μg/ml); ○——○, Pronase (150 μg/ml).

were treated with streptomycin and shown by observation of the ratio of their absorbancy at 280 mμ and 260 mμ to contain less than 3% nucleic acid, were rapidly inactivated in the absence of protective agents. A similar inactivation of trypsin by nucleic acid has been reported (10).

TABLE 1

Effect of substrates and inhibitors on the inactivation of Escherichia coli dihydrofolate reductase by Pronase

Compound	Percent of protection at concentration (M)			
	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Dihydrofolate	50	87	90	90
Folate	0	10	40	70
NADP	34	57	65	90
NADPH	50	70	80	85
NAD	—	—	—	0
NADH	—	—	—	0

Other substrates and cofactors have been tested in assay systems similar to that used to study protection by dihydrofolate. The results are summarized in Table 1. Folic acid also appeared to protect the reductase but, compared to dihydrofolate, 100 times as much was required to produce a similar degree of protection. It has not been possible to demonstrate the reduction of folic acid by preparations of *E. coli* dihydrofolate reductase (7), but these data suggest that folate is capable of binding that enzyme at least weakly.

NADPH, which acts as the reduced cofactor for the reductase, provided almost the same degree of protection as did dihydrofolate. When present at 10^{-4} M, 80% of the reductase remained at the end of the incubation period whereas only 50% remained when the concentration of NADPH was 10^{-6} M. It appears that concentrations equal to or greater than those necessary to achieve the maximum reaction velocity (as measured in the spectrophotometric assay) are required for complete protection. Surprisingly, NADP, one of the reaction products, was about as effective a protecting agent as NADPH. The competence of NADP as a protective agent was not due to a generalized lack of specificity in the binding of pyridine nucleotides. NAD and NADH were both ineffective as protectors at 10^{-3} M even though NADH was 25% as effective as NADPH as a cofactor for the

reduction of dihydrofolate in the spectrophotometric assay system.

Although ineffective as a protective agent, NAD did not accelerate the rate of destruction of dihydrofolate reductase by Pronase. As shown in Fig. 2 the activity of a system containing *E. coli* dihydrofolate reductase and NAD (10^{-4} M) but lacking in Pronase remained stable over the entire 90-min incubation period, while essentially all activity was lost when the reductase was incubated in the presence of Pronase (20 μg/ml). The addition of either 10^{-4} M NAD or 10^{-6} M NAD to the system containing reductase and Pronase (20 μg/ml) caused no significant change in the rate of destruction of dihydrofolate reductase. It is probable that NAD does not bind the dihydrofolate reductase of *E. coli* to any appreciable extent.

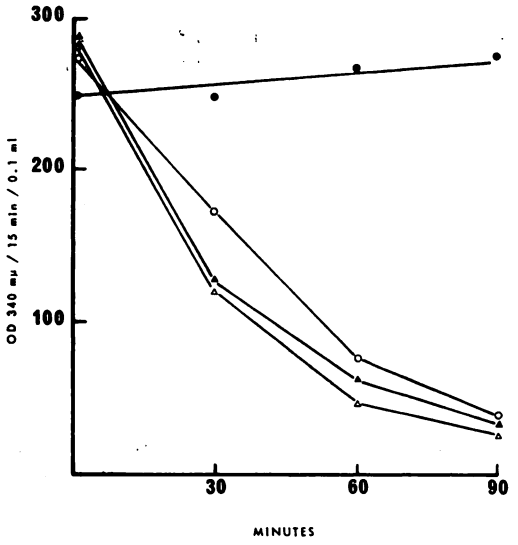


FIG. 2. *Inability of NAD to accelerate the inactivation of Escherichia coli dihydrofolate reductase by Pronase*

All systems contained *E. coli* dihydrofolate reductase and buffer as described in Methods and Materials plus the following additions: ●, NAD (10^{-4} M); ○, NAD (10^{-4} M) + Pronase (20 μg/ml); ▲, Pronase (20 μg/ml); △, NAD (10^{-4} M) + Pronase (20 μg/ml).

Since NADP served as a protector, it was of interest to know whether the other reaction product, tetrahydrofolate, possessed similar protective properties. However, the lability of this compound raised the possibility that the product(s) of its oxidation (e.g., folate, dihydrofolate or its components) might themselves protect or even interfere with protection. For these reasons it was decided to test the more stable N^5-formyl derivative of tetrahydrofolate, calcium leucovorin. The results of these experiments are shown in Table 2. Leucovorin

TABLE 2

Effect of calcium leucovorin and component moieties of dihydrofolate on the inactivation of E. coli dihydrofolate reductase by Pronase

Compound	Percent of protection at concentration (M)			
	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Ca leucovorin[a]	12	45	89	90
$CH_2OH \cdot Ptd \cdot H_2$[b]	—	—	0	0
pABG[c]	—	—	0	0
$CH_2OH \cdot Ptd \cdot H_2$ + pABG	—	—	0	0
Glutamate	—	—	0	0

[a] N^5-Formyltetrahydrofolate.

[b] 2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine.

[c] *p*-Aminobenzoylglutamate.

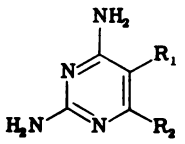
provided almost complete protection at concentrations of 10^{-3} and 10^{-4} M and 12% protection at 10^{-6} M. A comparison of the molar concentrations of both compounds required to provide approximately 50% and 90% protection showed leucovorin to be approximately one-tenth as potent a protector as dihydrofolate.

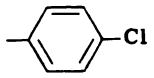
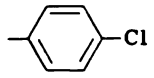
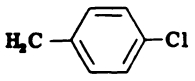
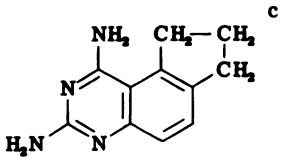
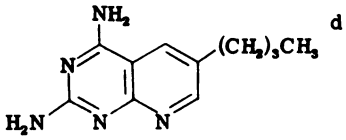
On the other hand, no component moiety of dihydrofolate that was tested showed any ability to protect. The 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine and *p*-aminobenzoylglutamate both failed to protect when tested either alone or in combination (at 10^{-3} and 10^{-4} M). Glutamate was also inactive at these same concentrations. None of the compounds shown in Table 1 or 2 had any effect on the ability of Pronase to hydrolyze casein.

The finding that substrates and cofactors protect dihydrofolate reductase suggested experiments to determine whether tightly bound inhibitors of this enzyme might

TABLE 3

Substituted diaminoheterocycles as protectors of microbial dihydrofolate reductase



	R_1	R_2	50% Inhibitory conc. (M)	Tested conc. (M)	Percent of protection
(1)	 [a]	H	1.2×10^{-5}	2.0×10^{-4}	100
(2)	 [a]	C_2H_5	2.5×10^{-6}	7.5×10^{-5}	99
(3)	 [b]	C_2H_5	3.8×10^{-6}	4.0×10^{-5} 1.0×10^{-6}	93 0
(4)	 [c]		1.0×10^{-6}	1.0×10^{-7}	81
(5)	 [d]		5.0×10^{-7}	1.0×10^{-5} 5.0×10^{-6}	82 0

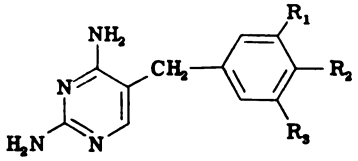
[a–d] Structures 1 and 2 are from Russel and Hitchings (11); structure 3, from Falco *et al.* (12); structure 4, from K. Ledig, E. Falco, and G. Hitchings, in preparation; and structure 5, from S. Harlbert and B. Valenti, *J. Med. Chem.* in press (1968).

possess a similar capacity. A variety of diamino heterocycles was studied, and the results are shown in Table 3. Compounds 1 and 2, 5-*p*-chlorophenyl-substituted diaminopyrimidines (11) proved to be extremely effective protectors when tested at 17 and 30 times their respective 50% inhibitory concentrations. The 5-*p*-chlorobenzyl-6-ethyl derivative (compound 3) (12) was an effective protective agent when tested at 10 times its 50% inhibitory concentration, but showed no activity at 1/400 of that value (i.e., when the enzyme was not saturated with the drug). In addition, compound 4 (13), 2,4-diamino-5,6-trimethylenequinazoline, and compound 5, 2,4-diamino-6-butylpyridopyrimidine, proved effective protective agents when present at concentrations greater than 10 times their 50% inhibitory concentration, even though their condensed ring systems differed significantly from each other and from the diaminopyrimidines.

Since the 5-benzyl diaminopyrimidines contain several compounds of interest in antibacterial chemotherapy, this series was

TABLE 4

Substituted diaminobenzylpyrimidines[a] as protectors of microbial dihydrofolate reductase



	R_1	R_2	R_3	50% Inhibitory conc. (M)	Tested conc. (M)	Percent of protection
(1)	H	OCH_3	H	1.1×10^{-6}	3.0×10^{-5}	100
(2)	OCH_3	OCH_3	H	2.0×10^{-7}	3.0×10^{-6}	93
					1.0×10^{-8}	0
(3)	OCH_3	H	OCH_3	5.6×10^{-8}	1.5×10^{-6}	90
(4)	OCH_3	OCH_3	OCH_3	5.0×10^{-9}	1.0×10^{-7}	0

[a] See Roth *et al.* (13).

examined in greater detail. The results are shown in Table 4. The *p*-methoxy-substituted compound (No. 1) (12) proved an excellent protector at 30 times its 50% inhibitory concentration. Similar results were obtained with the dimethoxy compound (No. 2) and again protection was lost when the concentration was lowered to less than one-twentieth of the 50% inhibitory concentration. The dimethoxy compound substituted in both meta positions (No. 3) also showed strong protection when tested at a saturating concentration.

In spite of the protection afforded to the reductase activity by the benzylpyrimidines, it was still possible that a portion of the enzyme had been cleaved without detectable loss of activity. In order to clarify this point, an aliquot of dihydrofolate reductase was treated with Pronase in the presence of a protective inhibitor, and its Sephadex G-75 elution profile was compared with that of the untreated reductase in order to establish the equivalence of the enzyme in both cases.

The experiment was conducted in the following manner: 2,4-diamino-5-(3',4'-dimethoxybenzyl)pyrimidine was added to an aliquot of *E. coli* dihydrofolate reductase to a final concentration of 3×10^{-6} M. One milliliter of the resulting solution was added to a 2×25 cm column containing Sephadex G-75 previously equilibrated with 1 mM EDTA. The enzyme was eluted with 1×10^{-3} M EDTA and collected in 1.0 ml fractions in the cold. After completion of this fractionation, a second solution of reductase and inhibitor was prepared exactly as before except for the addition of 200 μg of Pronase per milliliter. This system was allowed to incubate at room temperature for 4 hr (a control system containing no protective inhibitor lost all activity in 1 hr), and then 1.0 ml was placed on the same Sephadex G-75 column previously used. Once again the column was eluted with 1 mM EDTA and 1.0 ml fractions were collected. The results are shown in Fig. 3.

The upper portion of the figure (a) shows the system containing reductase, inhibitor, and Pronase. There were two main peaks containing material absorbing at 280 mμ. The smaller peak at the left (solid line) contained undigested reductase protein while the larger peak to the right contained the inhibitor and degraded protein. Reductase activity, indicated by the broken line, appeared in the first small peak. In the lower figure (b) the smaller and larger peaks again contained reductase protein and inhibitor, respectively.

Comparison of the upper and lower figures shows that in both cases reductase activity first appeared in tube No. 10 and peak reductase activity appeared in tube

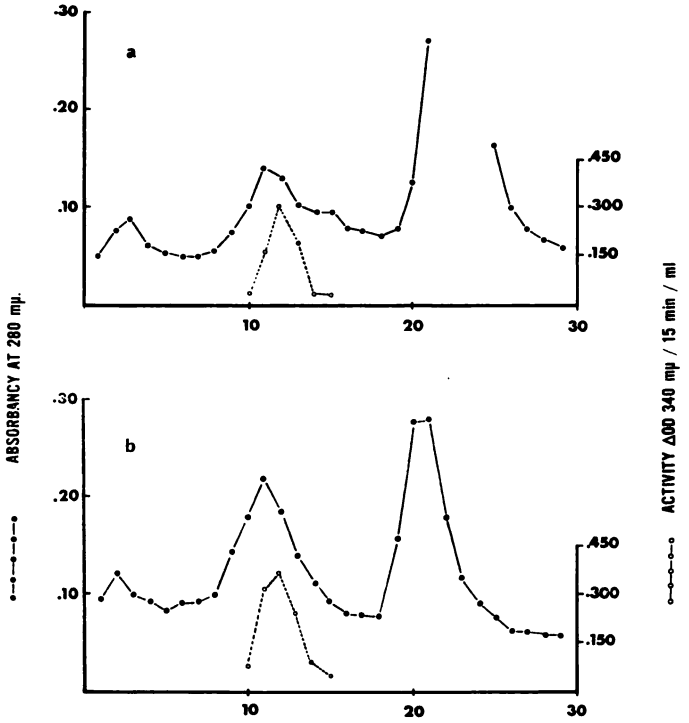


FIG. 3. *Comparison of the elution profile of dihydrofolate reductase and 2,4-diamino-5-(3',4'-dimethoxybenzyl)pyrimidine from Sephadex G-75*

(a) Enzyme treated with Pronase. (b) Untreated enzyme. See text for experimental details.

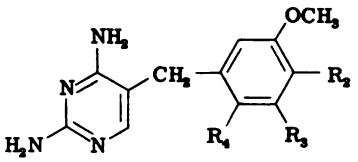
No. 12 regardless of whether the enzyme had been preincubated in the presence of Pronase. Under these experimental conditions it should have been possible to detect differences of greater than 10% in the size of the untreated and Pronase-treated reductase. Thus, it appears that proteolytic digestion of large areas of the reductase in the presence of the inhibitor is excluded, although loss of up to 21 amino acids might have escaped detection (assuming the molecular weight was 100 per amino acid residue and 21,000 for *E. coli* dihydrofolate reductase).

Experiments with the trimethoxybenzylpyrimidine, Table 4 (No. 4), trimethoprim, produced the major surprise of this study. No protection was afforded by saturating concentrations of the trimethoxybenzyl analog despite its extremely close structural similarity to the other compounds tested. After repeated experiments showed that this result was not due to experimental error, it was decided to investigate the extent of this effect in a series of close structural analogs of trimethoprim. The results of this study are shown in Table 5. The 2'-bromo trimethoprim (compound 1) also showed no protection when tested at 30 times its 50% inhibitory concentration. The same was true when there was substitution of the 4'-position with groups larger than methoxyl, such as butoxyl (compound 2) and allyloxyl (compound 3). Replacement of one of the metamethoxyl groups by bromine gave a compound (No. 4) which also was nonprotective. However, substitution of a chlorine atom in the same position (compound 5) resulted in a compound with a high degree of protective activity. Thus a very subtle and critical size requirement in this location is indicated.

Since the completion of the experiments described in this report we have obtained preparations of dihydrofolate reductase that are 65% pure. Using these highly

TABLE 5

Substituted diaminobenzylpyrimidines as protectors of microbial dihydrofolate reductase



	R_2	R_3	R_4	50% Inhibitory conc. (M)	Tested conc. (M)	Percent of protection
(1)	OCH_3	OCH_3	Br	2.8×10^{-8}	9.0×10^{-7}	0
(2)	OC_4H_9	OCH_3	H	1.0×10^{-8}	3.0×10^{-7}	0
(3)	$OCH_2CH=CH_2$[a]	OCH_3	H	5.2×10^{-9}	1.5×10^{-7}	0
(4)	OCH_3	Br	H	2.0×10^{-8}	6.0×10^{-7}	0
(5)	OCH_3	Cl	H	4.0×10^{-8}	9.0×10^{-7}	81

[a] To be published. All other compounds: see Roth *et al.* (13).

purified preparations the critical protection experiments involving 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine, Table 3, compound 2, and 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine, Table 4, compound 4, were repeated. The data obtained with the 65% pure enzyme were in complete agreement with those reported here for the 1% pure enzyme. In addition, determinations of the concentration of drug required to produce a 50% inhibition of the reductase have given consistent results from one preparation to another provided the purification was carried far enough to ensure the removal of NADPH oxidizing enzymes.

DISCUSSION

The mechanism by which substrates, cofactors, and inhibitors protect dihydrofolate reductase against proteolysis has not yet been established. The experiments of Hakala and Suolinna (5) have shown that dihydrofolate reductase of Sarcoma 180 cells is protected by substrates and cofactors from the action of trypsin, subtilisin, elastase, and carboxypeptidase B as well as *p*-chloromercuribenzoate, iodoacetamide, Versene, and *o*-phenanthroline. The results presented here and those of Hakala and Suolinna show clearly that, despite differences between them, both microbial and mammalian enzymes can be protected from enzymatic agents which possess a variety of different loci of attack.

The catalytic site(s) occupied by dihydrofolate (M.W. = 440) and NADPH (M.W. = 800) must constitute only a small fraction of the total reductase molecule (M.W. = 21,000). Even the catalytic site itself is probably not completely filled by protective agents such as inhibitors of the diaminopyrimidine class (M.W. = ca. 250) which lack the entire *p*-aminobenzoylglutamate portion of the substrate molecule. Unless considerable portions of the reductase molecule can be cleaved off without loss of activity, it is difficult to see how substrates and cofactors can prevent the destruction of the enzyme by protecting only those bonds they directly shield. An alternative possibility is that transition to the protected state involves more than just the active site.

On the basis of previous studies demonstrating protection against proteolysis (1) it was not unreasonable that dihydrofolate and NADPH should protect. However, since the equilibrium of the reductase reaction strongly favors the production of tetrahydrofolate, it is interesting to note that the reaction product NADP is an effective protective agent. This result is consonant with the finding of Perkins and Bertino (14), based on fluorometric studies, that NADP binds the dihydrofolate reduc-

tase of murine lymphoma ($K_D = 9 \times 10^{-7}$ M). These workers (14) also have provided evidence that the dihydrofolate reductase forms complexes with either the substrate or cofactor in the absence of the other coreactant. In our studies tetrahydrofolate also appears to bind and protect the reductase, but it is difficult to exclude the possibility that dihydrofolate is nonenzymatically generated during the course of the protection experiment. If both reaction products bind the reductase, this fact will have to be considered in any explanation of the reaction equilibrium, which is strongly toward tetrahydrofolate. The data presented here, which show that either substrate or cofactor alone protects, support a reaction mechanism involving random addition of substrates.

The most remarkable aspect of this study was the finding that certain diaminobenzylpyrimidines which tightly bind and strongly inhibit the bacterial reductase do not provide protection against inactivation by Pronase. At present it is not known whether those compounds substituted in the meta and para positions are unique in their failure to protect. These nonprotective compounds are all powerful inhibitors possessing in common 50% inhibition values of less than 3×10^{-8} M.

The possible physiological significance of the protection effect is of some interest. The findings reported above invite a reexamination of the cellular role of enzyme inhibitors that show protective ability. In the process of inhibiting their target enzyme, they may simultaneously allow its ultimate increase with unpredictable consequences for the survival of the affected cell.

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