Volume 6, No. 6, November, 1970
Printed in U.S.A.

MOLECULAR PHARMACOLOGY, 6, 617-220

Reduced Nicotinamide Adenine Dinucleotide Phosphate-Dependent Binding of Competitive Inhibitors to Dihydrofolate Reductase

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(Received June 19, 1970)

SUMMARY

The enzyme dihydrofolate reductase (EC 1.5.1.3), which catalyzes the reduced pyridine nucleotide-dependent reduction of dihydrofolate to tetrahydrofolate, is inhibited by 6-*N*-ω-(*N*-ethyl-*N*-2-chloroethyl)propyl-2,4,6-triamino-5-(dichlorophenylazo)pyrimidines acting as active site-directed irreversible inhibitors. A requirement for the binding of these inhibitors to the enzyme prior to alkylation is the presence of NADPH. Apparently the binding of NADPH to the enzyme causes a conformational change in the protein that makes the substrate-binding site accessible to either the substrate or a competitive inhibitor.

INTRODUCTION

It has recently been shown (1) that the activation of chicken liver dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3) by urea requires the presence of NADPH. Apparently, the binding of NADPH at the active site of the enzyme causes a conformational change in the protein. Perkins and Bertino (2) have suggested that the inhibition of dihydrofolate reductase by triamterene (6-phenyl-2,4,6-triaminopteridine) and methotrexate is attributable to the binding of the inhibitor to the enzyme-NADPH complex. It was therefore of interest to determine to what extent the occupancy of the NADPH-binding site by NADPH influences the binding of either dihydrofolate or a competitive inhibitor to the enzyme. The use of an active site-directed irreversible inhibitor permits the determination of the extent of binding at the dihydrofolate binding site in the presence and absence of NADPH.

EXPERIMENTAL PROCEDURE

Dihydrofolate reductase was prepared from rat liver, mouse liver, mouse intestine, and the Ehrlich ascites tumor by the procedure of Freudenthal and Hebborn (3). NADPH and folic acid were obtained from Sigma Chemical Company. Dihydrofolate was prepared from folic acid by the dithionite method of Futterman and Silverman (4) as modified by Blakely (5). Irreversible, active site-directed inhibitors of dihydrofolate reductase were 6-*N*-ω-(*N*-ethyl-*N*-2-chloroethyl)propyl - 2,4,6 - triamino - 5 - (dichlorophenylazo)pyrimidines (see Table 1). These compounds were synthesized by Dr. D. J. Triggle of this department.[2]

The reduction of dihydrofolate by dihydrofolate reductase was measured spectrophotometrically at 340 mμ, using a Gilford model 2000 attachment to a Beckman DU spectrophotometer. A decrease in absorbance occurs when NADPH and dihydrofolate are

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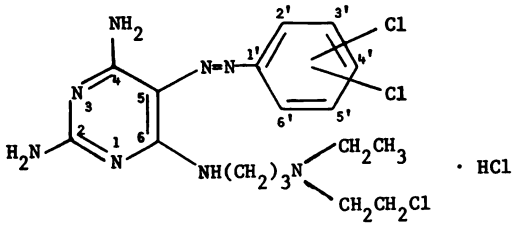
[2] D. J. Triggle, manuscript in preparation.

TABLE 1

Incubation time required for 50% inactivation of dihydrofolate reductase



The inhibitor concentration was 10 μM in the presence of 0.1 μM NADPH at 25°. For details, see EXPERIMENTAL PROCEDURE.

Compound	Dichloro Substituent positions	Time required for 50% inactivation of mouse tissue enzymes		
		Liver	Intestine	Ehrlich ascites
		min	*min*	*min*
1	2′,6′	34	22	60
2	2′,3′	8	8	4
3	2′,4′	36	20	60
4	2′,5′	38	23	45
5	3′,4′	35	22	40
6	3′,5′	40	19	29

TABLE 2

K_i values of competitive irreversible inhibitors for mouse dihydrofolate reductase

K_i values were determined by the graphical method of Freudenthal (7), in which $1/v$ is plotted against $1/S$.

Compound	Liver	Intestine	Ascites
	μM	μM	μM
1	0.6	0.2	0.3
2	0.1	0.1	0.2
3	1.1	1.3	1.8
4	2.6	2.1	3.4
5	0.8	0.6	0.5
6	0.3	0.3	0.5

converted to NADP and tetrahydrofolate, respectively (6). The standard assay mixture in the cuvette contained 0.05 M potassium phosphate buffer (pH 7.4), 0.1 mM NADPH, and 0.2 mM dihydrofolate. The enzyme preparation used in this study had a specific activity of 0.82 unit/mg of protein, where 1 unit is defined as that amount of enzyme catalyzing the reduction of 1 μmole of dihydrofolate per minute under the standard assay conditions described.

The rate of inactivation of dihydrofolate reductase in the presence or absence of NADPH was determined as follows. The incubation mixture without NADPH contained 1.6 ml of enzyme solution, 0.2 ml of water, and 0.2 ml of an inhibitor dissolved in water. For incubation in the presence of NADPH, 0.2 ml of 5 mM NADPH was substituted for the water. At suitable time intervals, 0.2-ml aliquots were withdrawn and added to a cuvette containing the remainder of the standard assay mixture. Aliquots from the incubation mixture without NADPH were added to 0.4 ml of 0.125 M potassium phosphate buffer (pH 7.4), 0.3 ml of 0.66 mM dihydrofolate solution, and 0.1 ml of 1.0 mM NADPH solution. Aliquots from the incubation mixture with NADPH were added to 0.3 ml of 0.66 mM dihydrofolate solution and 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4. All incubations were carried out at 25°.

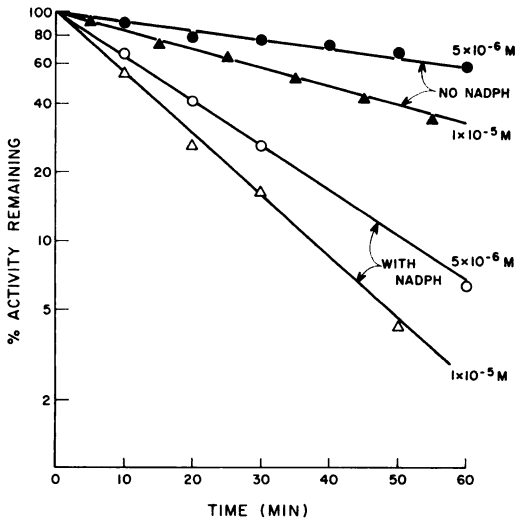


FIG. 1. *Inactivation of rat liver dihydrofolate reductase by compound 2*

The concentration of inhibitor was varied in the presence and absence of NADPH. The details of the procedure are described in EXPERIMENTAL PROCEDURE.

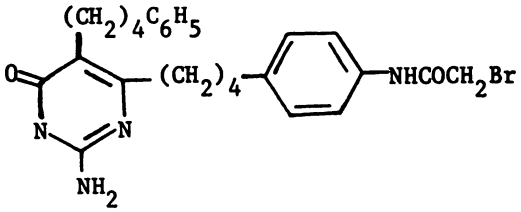
RESULTS

Incubation of dihydrofolate reductase with both NADPH and the inhibitors resulted in inactivation of the enzyme. The times required for 50% inactivation of the enzyme prepared from three mouse tissues are shown in Table 1. Five of the six isomers were comparable in their rates of inactivation of the enzyme, but compound 2 produced a more rapid rate of inactivation of the enzyme from all three tissues. This difference cannot be explained on the basis of a significantly greater affinity of compound 2 for the enzyme, because there is no correlation between K_i value and rate of enzyme inactivation (see Table 2). In addition, the alkylating activities of the six isomers, determined using 4-nitrobenzylpyridine (8), were similar. The inactivation of the enzyme was shown to be irreversible by passing the enzyme-inhibitor complex through a Sephadex G-75 column. That the inhibitor did not dissociate from the enzyme was regarded as a failure to increase specific activity of the enzyme obtained from the Sephadex column.

Omission of NADPH from the incubation of dihydrofolate reductase with an inhibitor resulted in a marked reduction in the rate of inactivation. The inactivation of rat liver dihydrofolate reductase by various concentrations of compound 2 in the presence and absence of NADPH is shown in Fig. 1 and Table 3. A very similar effect was seen with each of the other five inhibitors.

DISCUSSION

Inactivation of dihydrofolate reductase by 2,4-diamino-5-arylazopyrimidines having an alkylating group substituted in position 6 has been shown to be irreversible in nature (9) and is the consequence of alkylation of the enzyme. The ability of either substrate or nonalkylating competitive inhibitors to protect the enzyme has been reported previously (9). The present study indicates that, for the enzyme from rat liver, NADPH must be bound to the enzyme before active site-directed inactivation can occur. This is in contrast to the observation of Baker and Jordaan (10) that NADPH protects dihydrofolate reductase against inactivation by an alkylating pyrimidol:



The data in Table 3 indicate that inactivation is the consequence of a nonspecific bimolecular reaction between the enzyme and the alkylating agent in the absence of NADPH. Doubling the concentration of compound 2 doubles the rate of inactivation of the enzyme. However, in the presence of NADPH, the increase in rate of inactivation is seen to depend on a specific site-directed alkylation of the enzyme, i.e., on the formation of an initial reversible enzyme-inhibitor complex. The fraction of the total enzyme present (E_t) as the EI complex, calculated from the formula

$$[EI] = \frac{[E_t]}{K_i/[I] + 1}$$

is 0.55, 0.71, and 0.83 at inhibitor concentrations $[I]$ of 5 μM, 10 μM, and 20 μM, respectively, when a K_i value of 40 μM is assumed for compound 2 for the rat liver enzyme. Consequently, the rate of inactivation is increased, as calculated, by a factor of 1.29 when the concentration of inhibitor is doubled from 5 μM to 10 μM, and by a factor of 1.17 when the concentration is doubled from 10 μM to 20 μM. The experimental values obtained are 1.35 and 1.1 for the respective

TABLE 3

Inactivation of rat liver dihydrofolate reductase by compound 2

Experimental details are described in the text.

Inhibitor concentration	Time required for 50% inactivation	
	−NADPH	+NADPH
μM	*min*	*min*
5	74	15.5
10	36.5	11.5
20	17	10.0

increases in concentration. There is sufficiently close agreement with the theoretical values to support the suggestion that the presence of NADPH permits the formation of a reversible enzyme-inhibitor complex prior to alkylation.

Available evidence suggests that NADPH binds to the enzyme and produces a conformational change which allows the inhibitor to bind at the active site. It has been shown that either dihydrofolate or cofactor, in the absence of the other coreactant, forms a complex with dihydrofolate reductase (2). In addition, certain heterocyclic inhibitors of the enzyme, by formation of an enzyme-inhibitor complex, are excellent protective agents against inactivation by the proteolytic enzyme Pronase (11). In contrast, the six inhibitors used in the present study bound to dihydrofolate reductase only after the prior formation of an enzyme-NADPH complex. The apparently conflicting evidence presented by Baker and Jordaan (10) for the alkylating pyrimidol may perhaps be explained on the basis of the conformations assumed by the different inhibitors on the enzyme surface and their relationship to the binding site for NADPH.

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