INACTIVATION OF DIHYDROFOLATE REDUCTASE IN VITRO BY BINDING TO MICROSOMAL MEMBRANES

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SUMMARY

Dihydrofolate reductase was inactivated when incubated with the 27,000 x g pellet of lymphoma. Inactive enzyme was shown to be bound to the microsomal fraction and could be reactivated by urea. No acid-soluble material was produced nor did any change in the molecular weight of the enzyme molecule occur.

INTRODUCTION: The folate analogue, amethopterin (Methotrexate ©) is an effective drug in the treatment of cancer and inhibits the enzyme, dihydrofolate reductase. In addition, it increases the total amount of dihydrofolate reductase both in the cells of patients and in cultured human cells treated with the drug (1,2). However, little is known about the regulation of the activity or turnover of dihydrofolate reductase (3).

To understand these processes better, we measured the inactivation of the enzyme in cell free systems. Radioactively labelled enzyme was used to see whether inactivation was accompanied by the production of acid-soluble material or by a change in the molecular weight of the enzyme. Column chromatography using Sepharose 2B was used to detect binding of the enzyme to subcellular membranes.

METHODS

Preparation of 27,000 x g pellet: Cells used were a subline (LM) of the L1210 lymphoma (4) and were passaged every six days in the ascites form, 4×10^6 cells being injected into BDF1 mice. After harvesting, the cells were washed in saline and swollen by suspending them at a concentration of 4×10^8 cells/ml in 0.02M Tris-HCl, pH 7.4. After 10 min at room temperature, the cells were homogenized in a Dounce homogenizer fitted with a loose pestle and the homogenate immediately brought to 0.25M sucrose, 0.02M Tris-HCl,

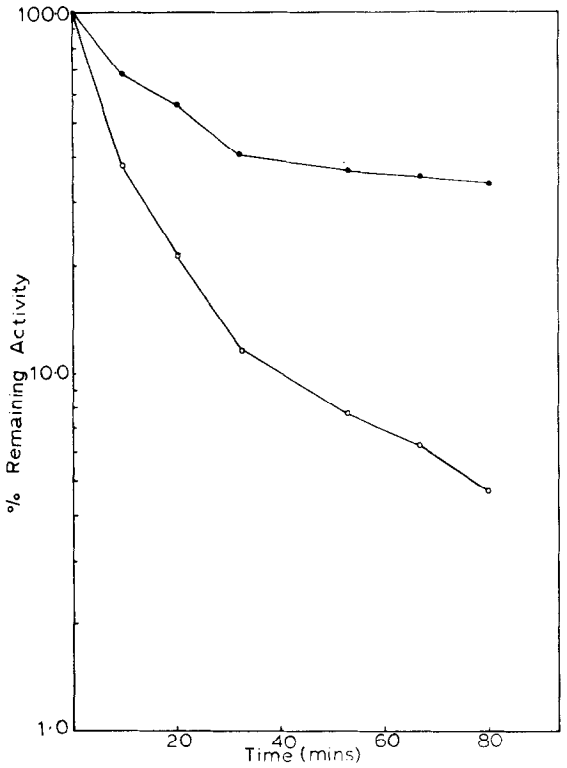

Fig. 1. Inactivation of dihydrofolate reductase during incubation with the 27,000 x g pellet of LM cells.

0.16 units of enzyme were incubated with 0.34 mg of protein from the 27,000 x g pellet in a final volume of 0.39 ml. Activities were measured by a continuous spectrophotometric assay (14).

Enzyme incubated with 27,000 x g pellet, o——o; enzyme incubated with boiled 27,000 x g pellet, ●——●.

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pH 7.4 by the addition of 1.0M sucrose in 0.02M Tris-HCl, pH 7.4. The homogenate was centrifuged at 600 x g for 15 min and the pellet discarded. The supernatant was then centrifuged at 27,000 x g for 20 min to obtain the 27,000 x g pellet. To prepare a mitochondria-rich fraction, lysosome-rich fraction and microsome-rich fraction, rather than the total 27,000 x g pellet, a different procedure was used. The 600 x g supernatant was centrifuged at 3,000 x g for 20 min for the mitochondria-rich fraction. The 3,000 x g supernatant was centrifuged at 8,000 x g for 20 min for the lysosome-rich fraction. The 8,000 x g supernatant was centrifuged at 27,000 x g for 20 min for the microsome-rich fraction. The 100,000 x g light microsomal fraction was prepared by centrifuging the 27,000 x g supernatant at 100,000 x g for 30 min. Each of the pellets was resuspended in 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 with five strokes of a Dounce homogenizer fitted with a loose pestle.

Cell fractions labelled with [^{3}H]-leucine were prepared from cells grown in mice which were injected with L-[4,5-^{3}H]-leucine. Mice were injected

Table 1

Production of acid-soluble material from [^{14}C]-labelled dihydrofolate reductase

	acid-soluble dpm	% acid-soluble
not incubated	734	5.15
	743	5.21
incubated with 27,000 x g pellet for 90 min	858	6.01
	866	6.07
incubated with boiled 27,000 x g pellet for 90 min	807	5.66
	773	5.42

Incubations contained 4.1 ug dihydrofolate reductase and 0.39 mg of protein from the 27,000 x g pellet in a final volume of 0.35 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. After 90 min 10% trichloroacetic acid was added to a final concentration of 5%. Bovine serum albumin, 1 mg/ml, was added as carrier protein. The samples were left at 4°C overnight, centrifuged and aliquots of the supernatant counted in Bray's scintillation fluid. The results given represent duplicate samples. Although the amount of acid-soluble material in the unincubated sample varied, similar results were obtained in three different experiments.

intraperitoneally with 25 μcuries zero, one, two, three and four days after introduction of the cells. The cells were harvested on the sixth day.

Radioactive labelling of dihydrofolate reductase: Dihydrofolate reductase was purified from the LM cells by affinity chromatography on a column of Sepharose-MTX as described by Gauldie and Hillcoat (5). The purified enzyme was methylated using [^{14}C]-formaldehyde and sodium borohydride (6) and dialyzed for 24 h against four changes of 1,000 volumes of 0.25M sucrose, 0.02M Tris-HCl, pH 7.4 to reduce acid-soluble counts to a low level.

Polyacrylamide-SDS gel electrophoresis: Polyacrylamide-SDS gel electrophoresis was by the methods of Fairbanks (7). Gels were sliced into 2 mm slices which were heated at 50°C for 2 h with 0.5 ml NCS. The radioactivity in each slice was counted after addition of 10 ml of Bray's scintillation fluid.

Chromatography of the products of incubation on a Sepharose 2B column: [^{14}C]-labelled dihydrofolate reductase was incubated with subcellular fractions of

Abbreviation: SDS, sodium dodecyl sulfate

LM cells for 2 h. Aliquots were applied to a column of Sepharose 2B (1 x 18 cm), previously equilibrated with 0.25M sucrose, 0.02M Tris-HCl, pH 7.4. The column was eluted at 4°C. Fractions of 0.65 ml were collected and the radioactivity in each determined.

RESULTS AND DISCUSSION

Dihydrofolate reductase was rapidly inactivated during incubation with the 27,000 x g pellet of LM cells at neutral pH. Inactivation was faster in the presence of the 27,000 x g pellet than in the presence of the boiled pellet (Fig. 1). Fifty percent of the enzyme activity was lost in 7 min, compared to 24 min in the boiled control.

The 27,000 x g pellet contains lysosomes, mitochondria and microsomes, each of which have been reported to contain proteases (8-11). Inactivation, however, does not appear to result from proteolysis since no acid soluble material was produced (Table 1), nor could any change in the mobility in SDS gels of the dihydrofolate reductase which remained in the 27,000 x g supernatant nor that which sedimented in the 27,000 x g pellet be detected. Nevertheless, the enzyme was strongly inactivated.

When [^{14}C]-labelled dihydrofolate reductase was incubated with the 27,000 x g pellet and then an aliquot of the 27,000 x g supernatant passed through a column of Sepharose 2B, inactive [^{14}C]-labelled material eluted in several fractions of high molecular weight as well as in a single active peak. In controls, which contained boiled 27,000 x g pellet, dihydrofolate reductase was found only in the single peak. Inactivation might result from binding of enzyme to fragments of the 27,000 x g pellet which remain in the 27,000 x g supernatant at the end of the incubation. In order to detect subcellular proteins in the eluate of the Sepharose 2B column, [^{3}H]-labelled proteins were used. It was shown that there was a good correlation between the peak of high molecular weight [^{14}C]-labelled enzyme and the [^{3}H]-labelled proteins of the microsomal fractions (Fig. 2).

The material in the high molecular weight fractions was inactive while that in the peak of lower molecular weight was active. These results suggest

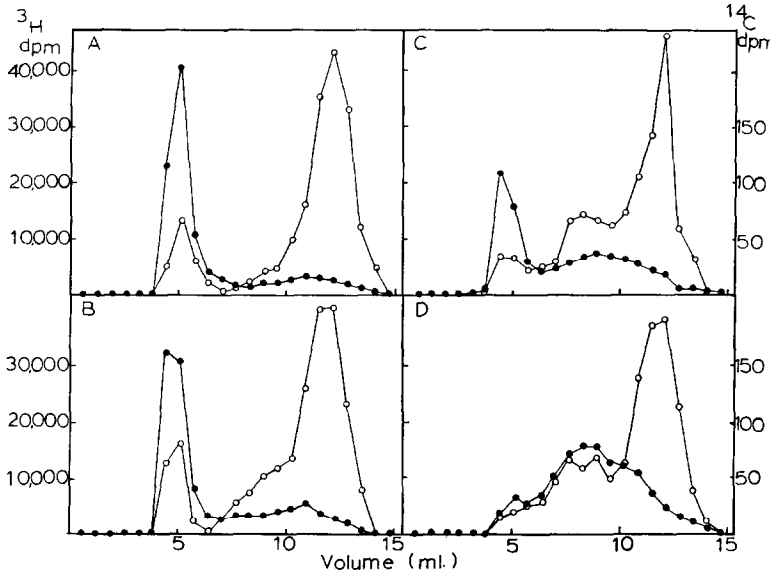

Fig. 2. Column chromatography of [^{3}H]-labelled subcellular fractions of LM cells and [^{14}C]-labelled dihydrofolate reductase on Sepharose 2B.

After incubation of 2.7 µg of dihydrofolate reductase with 0.47 mg of protein of the subcellular fraction at 37°C and in a final volume of 0.30 ml, 0.25M sucrose, 0.02M Tris-HCl, pH 7.4, 0.25 ml of the incubation mixture was applied to a column of Sepharose 2B. Closed circles represent [^{3}H] dpm and open circles [^{14}C] dpm.

(A) After incubation with mitochondria-rich fraction; (B) after incubation with lysosome-rich fraction; (C) after incubation with microsome-rich fraction; (D) after incubation with light microsomal fraction.

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that enzyme is inactive and bound to subcellular membranes. The greatest association of inactive [^{14}C]-labelled dihydrofolate reductase occurred with the microsomal membranes. Although it is possible that the inactive high molecular weight [^{14}C]-labelled material represents formation of inactive aggregates of enzyme catalyzed by the presence of subcellular fractions, the appearance of the material in several different fractions of high molecular weight which correspond to the position of the microsomal proteins suggests that binding to the microsomal membranes is the more likely explanation.

Our results do not tell us whether inactivation is the result of binding or whether enzyme is inactivated then bound. The failure to demonstrate a change in molecular weight of the dihydrofolate reductase molecule on poly-

acrylamide-SDS gels does not eliminate the possibility that cleavage of peptide bond(s) close to the end of the molecule has occurred. Such small changes in molecular weight would not be detected by the methods we have used. Kominami et al. (10) have recently described the inactivation of ornithine transaminase and shown the N-terminal amino acid of the enzyme to be altered while no change in molecular weight could be detected.

When the inactive [^{14}C]-labelled material of high molecular weight was eluted on a column of Sepharose 2B and treated with 8M urea and 1.2 mM mercaptoethanol, enzyme activity was restored. Calculations comparing the activity per dpm in fractions of lower molecular weight with the activity per dpm recovered in the fractions of high molecular weight after treatment with urea and mercaptoethanol indicated that the enzyme in these fractions was completely reactivated. In contrast, the active enzyme eluting in fractions of lower molecular weight was inactivated by this treatment. If incubations were treated with either 8M urea or 1.2 mM mercaptoethanol before they were passed through a column of Sepharose 2B, the enzyme activity increased in those incubations which contained only urea, but not in those which contained only mercaptoethanol. These results suggest that binding to the microsomal membranes may be by hydrophobic interactions. Such a mechanism is feasible since dihydrofolate reductase is known to contain hydrophobic regions (12).

Ballard and Hopgood (13) have recently reported that phosphoenolpyruvate carboxykinase (guanosine triphosphate) was inactivated in vitro by the microsomal fraction of liver extracts and that inactive enzyme was bound to the microsomal membranes.

Our results and those of Ballard and Hopgood (13) indicate that inactivation of enzymes by binding to microsomal membranes does occur but its significance in regulating enzyme activity in the intact cell remains to be determined.

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