Affinity Labeling of the 5-Methyltetrahydrofolate/Methotrexate Transport Protein of L1210 Cells by Treatment with an N-Hydroxysuccinimide Ester of \textsuperscript{3}H\textsuperscript{3}H]Methotrexate\textsuperscript{*}

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An N-hydroxysuccinimide ester of \textsuperscript{3}H\textsuperscript{3}H]methotrexate has been employed to covalently label a specific binding protein that resides in the plasma membrane of L1210 cells. Incorporation of radioactivity into this protein accounted for 55% of total cellular labeling, was half-maximal at a reagent concentration of 27 nM, and was blocked either by prior exposure to unlabeled reagent or by the addition of excess methotrexate. A role for this protein in methotrexate transport was supported by the observations that: (a) similar concentrations of reagent were required for both labeling of the binding protein and irreversible inhibition of transport; (b) the amount of labeled binding protein was comparable to observed levels of transport protein; (c) protection against labeling was afforded by thiamin pyrophosphate, a potent competitive inhibitor of methotrexate transport; and (d) labeling of the binding protein was not observed in a subline of L1210 cells that has a defect in the ability to transport methotrexate. The binding protein could be solubilized from the membrane by various ionic and non-ionic detergents and the covalent bond between the incorporated \textsuperscript{3}H\textsuperscript{3}H]methotrexate and the protein was stable to a variety of conditions, including high concentrations of mercaptoethanol and hydroxylamine and extremes of pH. The labeled protein fractionated as a nearly symmetrical peak on Sephacryl S-300 and it appeared as a single band (Mr = 36,000) after electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate.

Studies performed at low temperatures have provided evidence for a binding protein for folate compounds that is present on the external membrane surface of L1210 cells (1). The protein exhibits a high affinity for both 5-methyltetrahydrofolate (K\textsubscript{D} = 110 nM) and methotrexate (K\textsubscript{D} = 350 nM), and it appears to be involved in transport from similarities in the magnitude of K\textsubscript{D} values for substrate binding and corresponding K\textsubscript{V} values for their transport into the cell, and from comparable inhibition of 5-methyltetrahydrofolate binding and transport by various anions. Bound 5-\textsuperscript{14}C]methyltetrahydrofolate could be displaced rapidly (at 4°C) by excess unlabeled methotrexate, indicating that the complex formed between the substrate and binding site remains at the external membrane surface and is not internalized at low temperatures. The binding protein has not been purified to homogeneity, due primarily to the low levels of binder present in these cells (1 pmol/mg of total protein) (1, 2), and also to the lack of an effective assay for following the protein after solubilization from the membrane.

The transport of folate compounds into L1210 cells can be irreversibly inhibited by a variety of photoreactive and chemically reactive reagents. Effective compounds include sulfhydryl reagents (3, 4), 8-azido-AMP (5), 4,4'-diisothiocyanostilbene-2,2'-disulfonate (6), carbodiimide-activated folate compounds (2), and an N-hydroxysuccinimide ester of methotrexate (7, 8). The latter compound is the most effective inhibitor of this group, both in rate of inactivation and in concentration of reagent (20 nM) required for 50% inhibition of transport. Protection against inactivation was also observed with substrates added during exposure to this reagent (8), indicating that NHS-methotrexate\textsuperscript{1} interacts specifically at the binding site of the transport system and hence might be suitable for affinity labeling of the carrier protein. To investigate the latter possibility, the reagent was prepared using \textsuperscript{3}H\textsuperscript{3}H]methotrexate and then added to L1210 cells under conditions which are optimal for inactivation of methotrexate transport. It was observed that a stable covalent bond is formed between \textsuperscript{3}H\textsuperscript{3}H]methotrexate and a single protein residing in the plasma membrane. Various findings indicate further that the binding protein labeled by this procedure is a component of the transport system which mediates the uptake of methotrexate and other folate compounds into L1210 cells.

MATERIALS AND METHODS\textsuperscript{2}

RESULTS

Incorporation of \textsuperscript{3}H\textsuperscript{3}H]Methotrexate into L1210 Cells—Irreversible inhibition of methotrexate transport by NHS-methotrexate occurs optimally in cells exposed to the reagent at pH 6.8 for 5 min at 25°C (8). When these same treatment

\textsuperscript{1}The abbreviations used are: NHS-methotrexate, N-hydroxysuccinimide ester of methotrexate; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; NHS-folate, N-hydroxysuccinimide ester of folate.

\textsuperscript{2} Portions of this paper (including “Materials and Methods”) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 83M-3263, cite author(s), and include a check or money order for $1.80 per set of photocopies.
conditions were followed using reagent (20 nM) that had been prepared with $[^3H]$methotrexate, the cells were found to contain covalently bound $[^3H]$methotrexate. A major part of the label (89%) was found in the supernatant fraction of cells that had been exposed to 2% Triton X-100 for 10 min at 25°C (see "Materials and Methods"), while the remainder (11%) was associated with Triton-insoluble components. In addition, when excess methotrexate was added during the labeling step, the level of incorporated radioactivity in the soluble fraction was reduced nearly 3-fold, while no effect was observed on labeled constituents in the particulate fraction. It thus appeared that a Triton-soluble binding protein had reacted specifically with the reagent. The amount of labeled binding protein was 0.32 pmol/mg of protein, and it accounted for 55% of the total incorporated radioactivity. When the cells were grown to various densities and then treated with NHS-$[^3H]$methotrexate, the amount of binder/mg of protein was the highest in cells harvested during the early or mid log phase of growth (0.5-1.5 x 10^6/ml) (Fig. 1). The total amount of binding component, however, continued to increase with cells grown to densities in excess of 2 x 10^6/ml.

Site of $[^3H]$Methotrexate Incorporation—Subcellular fractions were prepared from intact cells in an effort to establish the location of the labeled binding protein. By comparing the amount of specifically incorporated $[^3H]$methotrexate/mg of protein, the binding protein was shown to be enriched in excess of 2-fold when the cells were converted to a crude membrane fraction (by Dounce homogenization), and by greater than 10-fold in plasma membranes isolated by sucrose density centrifugation (Table I). Plasma membranes derived from untreated cells also contained binding protein that could be labeled with NHS-$[^3H]$methotrexate, and the amount of specifically labeled protein was the same as in plasma membranes that had been labeled with reagent prior to cell fractionation. Conversely, specific labeling was not observed in Triton extracts of isolated plasma membranes. The latter extracts were also devoid of methotrexate-binding activity as determined by equilibrium dialysis (16 h, 4°C) of samples against Mg:HEPES:sucrose buffer containing 0.5 μM $[^3H]$methotrexate.

Concentration Dependence for Protein Labeling—The concentration dependence for the incorporation of $[^3H]$methotrexate into L1210 cell proteins is shown in Fig. 2. Labeling was highest in cells exposed to reagent alone, and the profile was approximately hyperbolic, while incorporation proceeded in a linear fashion and at a 3-fold lower level in cells that had been treated with reagent plus excess methotrexae. A similar reduction in labeling was also observed when the cells were exposed to reagent in the presence of thamin pyrophosphate (Fig. 2). A plot of the difference between $[^3H]$methotrexate incorporated into samples with no addition and with added methotrexate versus the concentration of NHS-$[^3H]$methotrexate (see Fig. 2) is shown in Fig. 3. A hyperbolic curve was obtained and a double reciprocal plot of the data (inset, Fig. 3) was linear and yielded a concentration for half-maximal labeling of 27 nM and an amount of binding protein of 0.7 pmol/mg of protein. Conversely, when the same labeling procedure was performed using a methotrexate-resistant subline of L1210 cells that has a defect in methotrexate transport (10), no specific labeling of a cellular binding component was observed. Nonspecific labeling, however, was approximately the same in both cell lines.

Effect of Pretreatment with NHS-methotrexate and Folate—Prior exposure of the cells to NHS-methotrexate prevented the subsequent labeling of the binding protein by NHS-$[^3H]$methotrexate (Fig. 4). Moreover, the concentration...
of NHS-methotrexate required for half-maximal reduction in labeling (20 nM) was essentially the same as the corresponding value (27 nM) obtained for half-maximal incorporation of radioactivity into the binding protein (Fig. 4). In contrast, treatment of the cells with NHS-folate at concentrations up to 500 nM had relatively little effect on labeling by NHS-[3H]methotrexate in the presence of 1 mM methotrexate served as the control.

Stability of the Labeled Protein—The covalent bond that had formed between the binding protein and [3H]methotrexate was stable to various incubation conditions (see "Materials and Methods" for experimental details). Release of incorporated [3H]methotrexate was minimal upon exposure of the labeled protein mixture either to heating (1 min, 100 °C) or to dialysis for 16 h at 23 °C against high concentrations of mercaptoethanol (100 mM) or hydroxylamine (500 mM), 0.1 N HCl, or 0.1 N NaOH.

Solubilization with Detergents—The relative effectiveness of various detergents in solubilizing the labeled binding protein is shown in Fig. 5. Release from isolated plasma membranes occurred most effectively in the presence of Triton X-100 and Nonidet P-40, with half-maximal extraction occurring at approximately 0.04%. A progressively lower ability to solubilize the binding protein was noted for CHAPS, cholate, and octylglucoside whose midpoint concentrations for extraction were 0.19, 0.46, and 0.50%, respectively. When the specific activities of various samples that had been solubilized with each detergent were compared (data not shown), none of the agents was found to extract the binding protein selectively.

Partial Purification of the Binding Protein—Labeled binding protein that had been extracted from isolated plasma membranes by Sephacryl S-300 chromatography of membrane proteins labeled with NHS-[3H]methotrexate in the absence and presence of protecting agent. Protein mixtures were derived from isolated plasma membranes of cells that had been labeled with NHS-[3H]methotrexate and extracted with Triton X-100. After precipitation with acetone, the protein was solubilized in 2% SDS, 10 mM sodium phosphate, 200 mM NaCl, 20 mM mercaptoethanol, pH 7.0, and then fractionated in the same buffer against 0.1% SDS, 10 mM sodium phosphate, 200 mM NaCl, 20 mM mercaptoethanol, pH 7.0, and then fractionated in the same buffer (at 23 °C) on a column (1.8 × 65 cm) of Sephacryl S-300. The void (exclusion) volume of the column (61 ml) was determined from the elution profile of blue dextran, while an inclusion volume of 151 ml was determined from the elution of [3H]leucine.

Fig. 6. Sephacryl S-300 chromatography of membrane proteins labeled with NHS-[3H]methotrexate in the absence and presence of protecting agent. Protein mixtures were derived from isolated plasma membranes of cells that had been labeled with NHS-[3H]methotrexate and extracted with Triton X-100. After precipitation with acetone, the protein was solubilized in 2% SDS, 10 mM sodium phosphate, 50 mM mercaptoethanol, pH 7.0, dialyzed for 16 h at 23 °C against 0.1% SDS, 10 mM sodium phosphate, 200 mM NaCl, 20 mM mercaptoethanol, pH 7.0, and then fractionated in the same buffer (at 23 °C) on a column (1.8 × 65 cm) of Sephacryl S-300. The void (exclusion) volume of the column (61 ml) was determined from the elution profile of blue dextran, while an inclusion volume of 151 ml was determined from the elution of [3H]leucine.
were combined, concentrated under vacuum to a small volume (0.1-0.2 ml), and then dialyzed at 23 °C for 16 h against 2% SDS, 62.5 mM Tris-HCl, 100 mM mercaptoethanol, 10% glycerol, pH 6.8, prior to electrophoresis (see "Materials and Methods"). Lane 1, protein sample stained with Coomassie blue; lane 2, autoradiogram of protein sample. Arrows indicate the position of protein standards which were (top to bottom) bovine serum albumin, ovalbumin, chymotrypsinogen, and L1210 dihydrofolate reductase.

membranes and then equilibrated in a buffer containing SDS was fractionated on a column of Sephacryl S-300 (Fig. 6). Bound radioactivity eluted in a generally symmetrical peak although a small shoulder appeared on the leading edge of the peak. Bound radioactivity was present in much smaller amounts in a parallel sample that had been labeled in the presence of excess methotrexate. The peak of radioactivity in the latter case was also broader than in the specifically labeled sample, and it had a maximum corresponding to a slightly lower molecular weight. Peak fractions from the Sephacryl column had a specific activity of approximately 14 pmol of bound [3H]methotrexate/mg of protein but, after SDS-polyacrylamide gel electrophoresis, were found to contain a multitude of proteins ranging in molecular weight from 30,000 to 100,000 (lane 1, Fig. 7). Autoradiography, however, revealed the presence of only a single radioactive band with an apparent molecular weight of 36,000 (lane 2, Fig. 7). Small amounts of nonspecifically labeled proteins were also present and could be visualized as a faint haze above the principal band.

**Discussion**

Folate-binding proteins have been shown to occur on the plasma membrane of various cells (1, 14-25), although in only a few instances has a biological function been established. A substantial number of these binders are probably involved in the transport of folate compounds into cells since folate, by virtue of its anionic charge, is highly water-soluble and hence shows little tendency to diffuse across cell membranes. Binding proteins which mediate folate transport have been isolated from Lactobacillus casei (15-17) and hog choroid plexus (23), although attempts at purification of analogous proteins from other sources have been hampered, in part, by the lack of a suitable assay after solubilization from the membrane.

The present study provides evidence that NHS-[3H]methotrexate can be employed to label the 5-methyltetrahydrofolate/methotrexate transport protein of L1210 cells. Cells exposed to this reagent contained covalently bound radioactivity that had been incorporated into a specific plasma membrane component (cf. Table I and Fig. 2), and a direct relationship could be established between the [3H]-labeled protein and the binding component of the transport system: (a) incorporation of [3H]methotrexate into cells (Fig. 3), the reduction in protein labeling by unlabeled reagent (Fig. 4), and inactivation of transport (8) each occur half-maximally at the same concentration of reagent (20-30 mM); (b) levels of the binding component (0.7 pmol/mg of protein) (Fig. 3) and of the transport protein (0.8-1.0 pmol/mg of protein) (1, 2) are essentially the same; (c) labeling of the binding protein with NHS-[3H]methotrexate (Fig. 2) can be prevented, not only by excess methotrexate, but also by thiamin pyrophosphate, an alternative anion substrate of the transport system (7, 9); and (d) the binding protein from a methotrexate-resistant subline of L1210 cells is not labeled by the reagent. The N-hydroxysuccinimide ester of folate, by comparison, is 500-fold less active in reacting with the binding protein (Fig. 4), in spite of the fact that folate binds to this system with only a 30-fold lower affinity than methotrexate (1). This finding suggests that the carboxyl groups on the glutamate portion of activated folate and methotrexate (and presumably also of the nonactivated substrates) have different orientations at the substrate-binding site.

The covalent bond that formed between the binding protein and NHS-[3H]methotrexate has not been identified, although stability measurements indicate that it is probably not an oxy-, thio-, or imidazole ester. The high stability of the complex is more characteristic of an amide bond which has been shown to result upon reaction of the folate transport protein of L. casei with an active folate ester (26). A consequence of the stable linkage between [3H]methotrexate and the L1210 binding protein is that the complex should be able to withstand a broad spectrum of conditions which might be encountered during purification. Preliminary fractionation experiments showed further that the binding protein can be solubilized from the plasma membrane with various detergents (Fig. 5) and that protein-bound radioactivity appears as a single, macromolecular component during both molecular sieve chromatography (Fig. 6) and SDS-polyacrylamide gel electrophoresis (Fig. 7). The latter observations in particular indicate that the binding protein is resistant to degradation by cellular proteases, does not readily form high molecular weight aggregates, and does not exhibit significant microheterogeneity, and therefore should be amenable to further purification. Isolation in a homogeneous form may be difficult, however, due to the low amount of binder present in these cells. Calculations from the binding activity in intact cells (1 pmol/mg of protein) and the theoretical binding capacity (28 nmol/mg of protein) for purified protein indicate that an enrichment of 28,000-fold would be required to achieve homogeneity. Three methotrexate-binding proteins have been isolated previously from Triton extracts of L1210 plasma membranes (21), although each of the latter proteins (M, = 56,000-67,000) differ substantially in molecular weight from the single binding protein identified in the present study (M, = 36,000). The labeled L1210 binding protein is much closer in size to a folate receptor (M, = 38,500) that has been isolated from human placenta (22).

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Supplemental Material to

Affinity-labeling of the 5-methylthiathyminelabeled methotrexate transport protein of L1210 cells by treatment with an anti-hydroxyspermine antibody of [3H]methotrexate

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MATERIALS AND METHODS

Chemicals: [3H]-[5,10-methotrexate (20 Ci/mmol) was obtained from Amersham, purified by triethylamine chromatography as described previously (1), and stored at 30°C in 10% ethanol. Other chemicals were obtained as follows: MEM, EC, DMSO, and o-ethylisocysteine, Cellgro, methotrexate, N-hydroxysuccinimide, triethylammonium chloride, Sigma; Triton X-100, D.I. water; and Kolhouse, Kocher Research Laboratories.

Preparation of affinity-labeled [3H]methotrexate. [3H]Methotrexate (4 nmol) was acidified by the addition of HCl (10 mmol), diluted under vacuum, and then converted to NIS-1-[3H]methotrexate by dissolving it in 1.2 ml of 100% dimethylformamide containing 20°C and 25°C methotrexate and incubating the mixture for 2 hr at 25°C. The reaction was usually employed immediately, although it could be stored for at least 3 hr at 4°C without loss in activity.

Preparation of L1210 cells with NIS-1-[3H]methotrexate. Parental L1210 mouse leukemia cells and methotrexate-resistant cell line with a defect in ability to transport methotrexate (10) were grown in suspension cultures as described previously (1), harvested during the late-growth phase of growth (1.5-3 x 10^6 cells/ml) washed with treatment buffer (20 mm HEPES-250 mm sucrose, pH 6.5 with MOPS), and suspended in the same buffer to a density of 10^5 cells. NIS-1-[3H]methotrexate was added to a final concentration of 20 mm (unless otherwise indicated), either to cells alone or to cells with added protecting agents, and the resulting mixtures were incubated for 1 hr at 25°C. Cells were then recovered by centrifugation at 2000 x g (15 min, 4°C), washed twice with HEPES-buffered saline (20 mm HEPES-140 mm NaCl-15 mm KCl-2 mm CaCl_2-2 mm MgCl_2-0.1 mm phenylphosphonic acid) to 1-2 x 10^4 cells.

Determination of incorporated [3H]methotrexate. Cells were collected and resuspended with 10 mm methotrexate were suspended to 20 mg protein/ml in HEPES-buffered saline and then digested by incubation in the presence of 20 Triton X-100 for 10 min at 37°C. After centrifugation at 10,000 x g (15 min, 4°C), the supernatant solution was collected, aliquoted to a final concentration of 50%, and the mixture was kept at 37°C for 30 min. The precipitated protein was then recovered by centrifugation, dissolved in 0.5 ml 250 mm sucrose phosphate, pH 7.0, and analyzed for radioactivity and protein.

Crude cell membranes. Labeled cells (10 mg protein/ml) that had been allowed to swell for 30 min at 4°C in lysis buffer were disrupted by homogenization in a micro-700 (ground homogenizer as described by Kozminski et al. (11)). The particulate fraction was then recovered, centrifuged at 20,000 x g (2 min, 4°C), precipitated with triton X-100, precipitated with triton X-100, and analyzed for incorporated radioactivity and protein as described above.

Plasma membranes. Crude cell membranes (15 mg protein/ml) that had been obtained by gentle homogenization (see above) were suspended in lysis buffer and applied to a discontinuous sucrose gradient consisting of 2 ml of 0.5 and 2.0 mmol of 10°C and 20°C sucrose in lysis buffer. After centrifugation at 20,000 x g (2 min, 4°C), the 200-300 fraction was collected, diluted 5-fold with lysis buffer, centrifuged at 4,000 x g, and resuspended in HEPES-buffered saline. Analysis for incorporated radioactivity and protein was then performed as described above.

Stability measurements on the total binding protein. Labeled cells were treated with NIS-[3H]methotrexate (30 ml), converted to plasma membranes, and extracted with 20 mm HEPES buffered saline (40°C above). After centrifugation, the supernatant fraction (containing approximately 1 mmol of acridine chromatographing protein) was distributed into 2.5-ml aliquots, and either buffer for 1 min at 100°C, held at 4°C during the pretreatment interval. Proteins in each of the samples were then precipitated with the addition of acetone (10%, recovered after centrifugation, reconstituted in 2.5 ml of 50% or 0.1 N HCl, or 0.1 N HCl, diluted to 100% methotrexate. Incubated radioactivity and protein were determined after an additional incubation for 1 hr at 25°C against the standard buffer. Non-specific labeling was determined in parallel samples that had been derived from cells exposed to receptors in the presence of 1 mm methotrexate.

Cell electrophoretography. Cells was electrophoretically at 140 V in 11 polyacrylamide gels containing 5% by the general procedure of Lowry at el. (17).

Autoradiography. Autoradiograms were prepared from SDS-slab gels containing 50,000-8,000 type of [3H]-labeled proteins. After electrophoresis, the gels were stained with coomassie blue (17), soaked in water (2 hr, 25°C) to remove residual acid, treated (3 hr, 25°C) with a trichloroacetic acid (40%, trichloroacetic acid, dried, and exposed to x-ray film (x-ray) for 14 days at -10°C.

Protein determination. Protein was determined by the procedure of Lowry et el. (11), employing bovine serum albumin as the standard.
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