# Affinity Labeling of the Folate–Methotrexate Transporter from *Leishmania donovani*†

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ABSTRACT: An affinity labeling technique has been developed to identify the folate–methotrexate transporter of *Leishmania donovani* promastigotes using "activated" derivatives of the ligands. These "activated" derivatives were synthesized by incubating folate and methotrexate with a 10-fold excess of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) for 10 min at ambient temperature in dimethyl sulfoxide. Preincubation of intact cells with nonradioactive "activated" folate or methotrexate at a concentration of 40 $\mu$M inhibited the capacity of wild-type cells to transport submicromolar concentrations of unmodified ligand. When intact wild-type (DI700) *Leishmania donovani* or preparations of their membranes were incubated with a 0.4 $\mu$M concentration of either "activated" [$^3$H]folate or "activated" [$^3$H]methotrexate, the radiolabeled ligands were covalently incorporated into a polypeptide with a molecular weight of approximately 46 000, as demonstrated by SDS–polyacrylamide gel electrophoresis. No affinity labeling of a 46 000-dalton protein was observed when equimolar concentrations of "activated" radiolabeled ligands were incubated with intact cells or membranes prepared from a methotrexate-resistant mutant clone of *Leishmania donovani*, MTXA5, that is genetically defective in folate–methotrexate transport capability [Kaur, K., Coons, T., Emmett, K., & Ullman, B. (1988) *J. Biol. Chem. 263*, 7020–7028]. However, some labeling of a 46 000-dalton protein was observed when MTXA5 cells were incubated with higher concentrations of "activated" ligands. Time course studies indicated that maximal labeling of the 46 000-dalton protein occurred within 5–10 min of incubation of intact cells with "activated" ligand. Inhibitors of folate–methotrexate transport, including dihydrofolate, tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and (*p*-aminobenzoyl)glutamate, blocked the incorporation of radiolabeled ligand into the 46 000-dalton polypeptide, while biopterin and pteroic acid, two pterins that did not block folate–methotrexate entry into wild-type *Leishmania donovani*, did not interfere with the labeling of the 46 000-dalton moiety. These studies provide biochemical evidence that the folate–methotrexate transporter of *Leishmania donovani* can be identified in crude extracts by an affinity labeling technique and serve as a prerequisite to further analysis of the transport protein by providing a vehicle for subsequent purification of this membrane carrier. Moreover, these investigations suggest that the affinity labeling technique using EDC-activated ligands may be exploitable to analyze other cell surface binding proteins in *Leishmania donovani*, as well as in other organisms.

*Leishmania donovani* is a species of parasitic protozoa that is the causative agent of visceral leishmaniasis, a disease that is often fatal to humans. *Leishmania* exist in two different life stages that can be distinguished on the basis of biochemical, morphological, and structural criteria. Inside the gut and salivary glands of their insect vectors, members of the phlebotomine sandfly family, *Leishmania* exist as the extracellular, motile, flagellated (promastigote) form. When an infected sandfly bites a human or animal, the promastigotes are engulfed by macrophages and cells of the reticuloendothelial system and are transformed to amastigotes, which are round, lack flagella, and are nonmotile.

The plasma membrane of *Leishmania* serves as the interphase between the parasitic metabolic machinery and the extracellular milieu and acts as both a barrier and a mediator for the entry of many small nutrients which are required for parasite survival and growth. Biochemical and immunological investigations have revealed the presence of multiple different proteins associated with the cell membrane (Dwyer, 1980; Lepay et al., 1983; Gardiner et al., 1984). Studies by Gottlieb and Dwyer (1981a,b, 1982), for example, have demonstrated the existence of various membrane-associated enzymes, including 5′-nucleotidase, 3′-nucleotidase, and acid phosphatase activities. Kinetic and biochemical investigations have indicated that leishmanial membranes contain various types of important transport systems, including those for hexoses (Schaeffer & Mukkada, 1976; Zilberstein & Dwyer, 1984, 1985), pentoses (Pastakia & Dwyer, 1987), amino acids (Zilberstein & Dwyer, 1985; Bonay & Cohen, 1983; Law & Mukkada, 1979), nucleosides (Hansen et al., 1982; Iovannisci et al., 1984; Aronow et al., 1987), and folate (Ellenberger & Beverley, 1987a,b; Kaur et al., 1988). Somatic cell genetic approaches have been particularly useful in identifying the multiplicity and substrate specificities of the leishmanial nucleoside transporters (Iovannisci et al., 1984; Aronow et al., 1987), properties which distinguish them from the well-characterized mammalian nucleoside transporter (Berlin & Oliver, 1975; Plagemann & Wohlhueter, 1980; Cohen et al., 1979).

Recently, Ellenberger and Beverley have isolated methotrexate-resistant mutants of *Leishmania major*, which are deficient in folate–methotrexate transport capacity (Ellenberger & Beverley, 1987). These workers demonstrated that

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the defect in folate–methotrexate entry is independent of another mechanism of methotrexate resistance in these organisms, i.e., gene amplification (Ellenberger & Beverley, 1987). Using a slightly different selective strategy, Kaur et al. (1988) have isolated in a single step a mutant clone of *Leishmania donovani* by virtue of its resistance to methotrexate toxicity. This cell line, MTXA5, is incapable of translocating both radiolabeled folate and methotrexate across its plasma membrane.

Biochemical and molecular analysis of transport proteins requires a mechanism for their purification. The purification of membrane binding or transport proteins from *Leishmania*, as well as from other organisms, has proven particularly intractable since transporters lose their biological function when removed from their hydrophobic cellular environment. Thus, it is important to develop appropriate "tags" for membrane proteins, which permit their detection after membrane solubilization. In order to identify functional proteins in intact cells or in crude extracts, radioactive molecules have been attached to specific proteins using both affinity and photoaffinity labeling techniques (Jakoby & Wilchek, 1977). Membrane proteins that have been identified by affinity and photoaffinity labeling protocols include ion channels (Ruoho & Kyte, 1977) and transporters for amino acids (Glover, 1977), biotin (Bayer & Wilchek, 1977), glucose (Carter-Su et al., 1982), and nucleosides (Young et al., 1983). Recently, a plasma membrane derived folate binding protein from murine L1210 cells has been identified by using a photoaffinity analogue of methotrexate (Price & Freisheim, 1987; Price et al., 1988).

In order to facilitate purification of the leishmanial folate–methotrexate transporter, we have exploited a procedure initially described and employed by Henderson and co-workers to inactivate the methotrexate transporter of L1210 cells (Henderson et al., 1980) and folate transport in *Lactobacillus casei* (Henderson & Potuznik, 1982). Using [$^3$H]folate and [$^3$H]methotrexate activated with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), we have developed a simple, specific, and inexpensive affinity labeling technique to identify the folate–methotrexate transporter of *Leishmania donovani*. This transporter has a molecular weight of approximately 46 000 and was not labeled by EDC-activated [$^3$H]folate or [$^3$H]methotrexate in MTXA5 cells genetically deficient in folate–methotrexate transport. This affinity labeling technique should facilitate protein purification and may be amenable to the identification, isolation, characterization, and purification of other functional membrane transporter proteins.

## Experimental Procedures

*Chemicals and Reagents.* [3′,5′,9(N)-$^3$H]Methotrexate (20 Ci/mmol) and [3′,5′,7,9(N)-$^3$H]folate (18 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Radiolabeled ligands obtained from the manufacturer were judged to be greater than 98% pure by the chromatographic system described by Silber et al. (1963) and were stored at −80 °C. Nonradioactive folate, methotrexate, dihydrofolate, tetrahydrofolate, 5-methyltetrahydrofolate, pteroic acid, biopterin, adenosine, inosine, HEPES, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were purchased from the Sigma Chemical Corp. (St. Louis, MO). Folinic acid (leukovorin) was obtained from Lederle Parenterals, Inc. (Carolina, Puerto Rico). Triton X-114 was bought from the Fluka Chemical Corp. (Ronkonkoma, NY). All other materials, chemicals, and reagents were of the highest quality commercially available.

*Cell Culture.* The promastigote (insect vector) form of clone 1S of the Sudanese strain of *Leishmania donovani* was grown in continuous culture at 26 °C in a humidified 10% $CO_2$ atmosphere in a completely defined growth medium originally described by Iovannisci and Ullman (1983). This medium, DME-L, uses Dulbecco's-modified Eagle's medium (Flow Laboratories, Mclean, VA) as the powder base and contains 5 mg/L hemin and 0.1 mM xanthine as a source of purine. DME-L contains 9.1 μM folate. Custom-made folate-deficient DME-L medium was prepared from folate-deficient Dulbecco's-modified Eagle's medium prepared by the University of California at San Francisco Cell Culture Facility. The DI700 parental strain is a wild-type clone of *Leishmania donovani* that was isolated in semisolid medium (Iovannisci & Ullman, 1983) containing 1% agar (Difco Laboratories, Detroit, MI). The MTXA5 strain was isolated from a mutagenized population of DI700 cells by virtue of its resistance to 1 mM methotrexate and is deficient in folate and methotrexate transport capability (Kaur et al., 1988). The MTXA5 cells were continuously monitored for retention of phenotype by incubating them in DME-L medium containing 1 mM methotrexate, a concentration that kills DI700 organisms (Kaur et al., 1988).

*Synthesis of "Activated" Folate and "Activated" Methotrexate.* The "activated" forms of folate and methotrexate were prepared essentially as described (Henderson et al., 1980). Briefly, the appropriate amount of folate or methotrexate was evaporated to dryness under a stream of nitrogen and mixed with a 10-fold excess of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) in dimethyl sulfoxide. The mixture was then incubated at 23 °C for 30 min unless otherwise stated.

*Affinity Labeling.* Intact organisms were washed with 10 mM HEPES, pH 7.4, containing 1 mM $MgCl_2$ and resuspended in wash buffer at a density of $5 \times 10^8$ cells/mL. Visual observation indicated that organisms remained viable and motile during this washing procedure. Typically, $10^8$ organisms were incubated for 10 min at 0 °C with 0.4 μM "activated" [$^3$H]folate (18 Ci/mmol) or 0.4 μM [$^3$H]methotrexate (20 Ci/mmol) unless otherwise indicated. Under the experimental manipulations, the concentration of dimethyl sulfoxide in the affinity labeling mixture was only 1%. After incubation with the "activated" ligand, cells were harvested by centrifugation and washed 3 times with 1.0 mL of the HEPES–$MgCl_2$ buffer, after which the cell pellet was lysed in 200 μL of Laemmli lysis buffer and sonicated for 10 s at 0 °C to minimize macromolecular aggregation. The affinity labeling experiments were performed in the absence of light to minimize destruction of the light-sensitive pterins.

*Transport Measurements.* The abilities of wild-type and mutant cells to transport 0.25 μM [$^3$H]folate (18 Ci/mmol) or 0.5 μM [$^3$H]methotrexate (20 Ci/mmol) were assessed by a modification of the rapid sampling kinetic protocol developed by Aronow et al. (1987) for their studies of nucleoside transport in *Leishmania donovani* promastigotes. Cells were harvested by centrifugation, washed with the HEPES–$MgCl_2$ buffer employed in the affinity labeling experiments, and resuspended in folate-deficient DME-L supplemented with 20 mM HEPES, pH 7.4, at a density of $5 \times 10^8$ cells/mL. Transport measurements were initiated by combining 100 μL of cell suspension with 100 μL of radiolabeled ligand in folate-deficient DME-L in Eppendorf microfuge tubes overlaying 150 μL of chemically inert dibutyl phthalate. The assays were terminated by centrifugation at 10000*g* for 30 s during which the cells were separated from the transport medium by sedimentation through the organic layer. The radioactive aqueous

layer was removed by aspiration and the dibutyl phthalate washed twice with 1.0 mL of water. The dibutyl phthalate was aspirated, the cell pellet lysed with 100 μL of 1% Triton X-100, and the radioactivity associated with the cell pellet quantitated by liquid scintillation spectrometry.

In order to assess the ability of "activated" ligands to interfere with cell transport capability, DI700 cells at a density of $5 \times 10^8$ cells/mL in HEPES–$MgCl_2$ were incubated with 40 μM concentrations of either nonradioactive "activated" methotrexate or "activated" folate for 10 min at 0 °C. As controls, parallel incubations of DI700 cells with either 40 μM folate for 40 μM methotrexate served as controls. Cells were washed carefully in folate-deficient DME-L in order to remove excess "activated" ligand or underivatized folate or methotrexate (controls) and resuspended in folate-deficient DME-L containing 20 mM HEPES, pH 7.4, at a density of $5 \times 10^8$ cells/mL, and their ability to transport folate or methotrexate was assessed by the rapid sampling procedure described above.

*Isolation of Integral Membrane Proteins.* Integral membrane proteins were partially purified by the method of Bordier (Bordier, 1981; Etges et al., 1985). Wild-type and MTXA5 *Leishmania* were extracted for 10 min at 0 °C in 10 mL of HEPES–$MgCl_2$ buffer containing 1% precondensed Triton X-114. In some experiments, cells had been subjected to the affinity labeling procedure prior to membrane extraction. The cell suspension was centrifuged at 50000*g* at 4 °C for 15 min and the supernatant layered over 15 mL of a 6% sucrose cushion in a 50-mL centrifuge tube. The solution was incubated at 30 °C for 3 min to induce aggregation of the Triton X-114 micelles. The suspension was centrifuged at 1000*g* for 10 min at ambient temperature during which the detergent-enriched integral membrane proteins sedimented through the sucrose cushion. Water-soluble proteins remained on the top of the 6% sucrose layer and were concentrated by lyophilization. The lyophilized protein fractions were redissolved in a volume of 1% Triton X-114 that was equal to the detergent-phase volume. If the extracted parasites had been prelabeled with "activated" ligand, the two phases were analyzed directly for covalently bound radioactivity by SDS–polyacrylamide gel electrophoresis. Otherwise, the two subcellular fractions from the sucrose sedimentation were incubated separately with the "activated" compounds for 10 min at 0 °C as described above and subjected to molecular weight analysis by SDS–polyacrylamide gel electrophoresis.

*SDS–Polyacrylamide Gel Electrophoresis.* Affinity-labeled proteins were electrophoresed on 12% polyacrylamide slab gels containing 1% SDS and 5 mM dithiothreitol as described (Laemmli, 1970). Gels were stained with Coomassie blue for 30 min, destained for several hours, and exposed to Fluorohance (New England Nuclear, Boston, MA) for 30 min. Gels were dried and fluorographed for 8–15 days.

## Results

*Affinity Labeling of Intact Cells.* In order to identify the folate–methotrexate transporter of *Leichmania donovani*, folate and methotrexate "activated" with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were incubated at a final concentration of 0.4 μM for 10 min at room temperature with intact wild-type and MTXA5 promastigotes, the latter genetically defective in folate and methotrexate transport capability (Kaur et al., 1988). The data in Figure 1 demonstrate that 0.4 μM concentrations of "activated" folate and "activated" methotrexate covalently labeled only a single protein in wild-type DI700 cells. This protein migrated as a sharp band in SDS gels with a molecular weight of approximately 46000. The data in Figure 1 also clearly indicate that

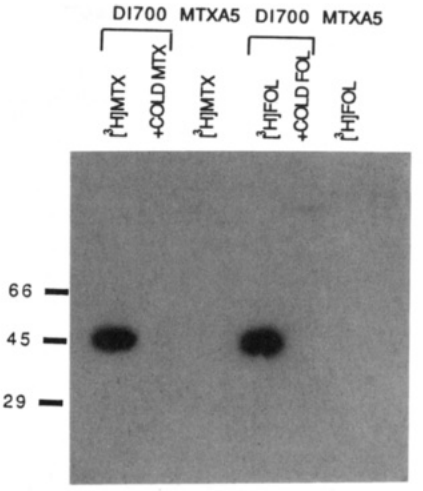


FIGURE 1: Affinity labeling of intact DI700 and MTXA5 cells with EDC-activated ligands. Intact wild-type and methotrexate-resistant *Leishmania*, the latter strain deficient in folate/methotrexate transport capability, were incubated with 0.4 μM either EDC-activated [$^3$H]methotrexate or EDC-activated [$^3$H]folate for 10 min at 0 °C. Where indicated for DI700 cells, a 100-fold excess of nonradiolabeled ligand was present during the affinity labeling procedure. The radiolabeled polypeptides were analyzed by SDS–polyacrylamide gel electrophoresis. The positions of the molecular weight standards of 29000, 45000, and 66000 are indicated at the left of the electrophoretogram.

no covalently labeled adduct was formed with any protein when the MTXA5 mutant cells were incubated with EDC-activated ligand. Moreover, 40 μM folate and 40 μM methotrexate prevented covalent attachment of the corresponding EDC-activated derivative to the 46000-dalton polypeptide in wild-type *Leishmania*. The concentration of EDC-activated ligand and the length of incubation of EDC-activated ligand with intact cells were maximized from experiments in which these parameters were treated as independent variables (data not shown); 1.0 and 4.0 μM concentrations of "activated" folate and methotrexate labeled the 46000-dalton polypeptide in wild-type cells to a greater extent than did 0.4 μM, but the specificity of the affinity labeling procedure was diminished considerably (data not shown). These higher concentrations of ligand labeled several other protein in both DI700 and MTXA5 cells, and some labeling of a 46000-dalton protein in mutant cells could also be detected.

*Affinity Labeling of Subcellular Fractions.* In order to demonstrate that the 46000-dalton polypeptide labeled in wild-type cells was associated with the leishmanial plasma membrane and not the cytosolic compartment, membrane and soluble fractions of DI700 and MTXA5 cells were prepared according to the method of Bordier (Bordier, 1981; Etges et al., 1985). As shown in Figure 2, incubation of plasma membrane fractions of DI700 cells with "activated" folate or "activated" methotrexate resulted in the specific labeling of the 46000-dalton protein, whereas no protein was affinity labeled in the MTXA5 mutant cells. Furthermore, no radioactive covalent adduct to any 100000*g* soluble protein in either wild-type or mutant cells could be detected after a 10-min incubation of cytosol with either "activated" ligand (Figure 2). Moreover, neither "activated" ligand labeled any soluble protein in either cell line when 100000*g* supernatants were prepared directly from extracted cells (data not shown).

*Inhibition of Transport by "Activated" Ligand.* The covalent linkage of a ligand to the active site of a transport protein would be expected to incapacitate the further function of that transporter. Therefore, the ability of intact wild-type *Leishmania* to transport either folate or methotrexate was

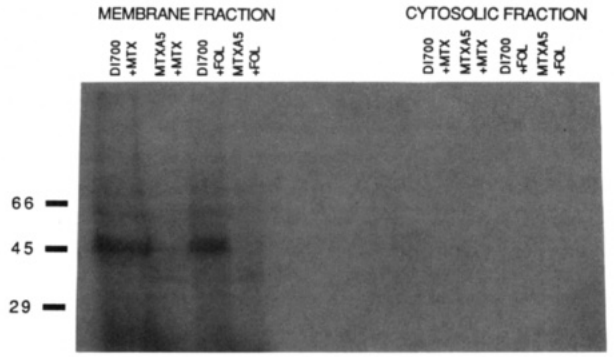


FIGURE 2: Affinity labeling of integral membrane and cytosolic fractions prepared from DI700 and MTXA5 cells. Integral membrane and soluble proteins were prepared from wild-type and mutant cells by the method of Bordier (1980). Fractions were labeled with either 0.4 $\mu$M EDC-activated [$^3$H]folate or 0.4 $\mu$M EDC-activated [$^3$H]-methotrexate in the absence or presence of a 100-fold excess of nonradiolabeled ligand for 10 min at 0 °C. The samples were dialyzed overnight to remove excess noncovalently bound ligand, analyzed by SDS–polyacrylamide gel electrophoresis, and subjected to fluorography, and the autoradiograms were developed after 1 week of exposure to the gel.

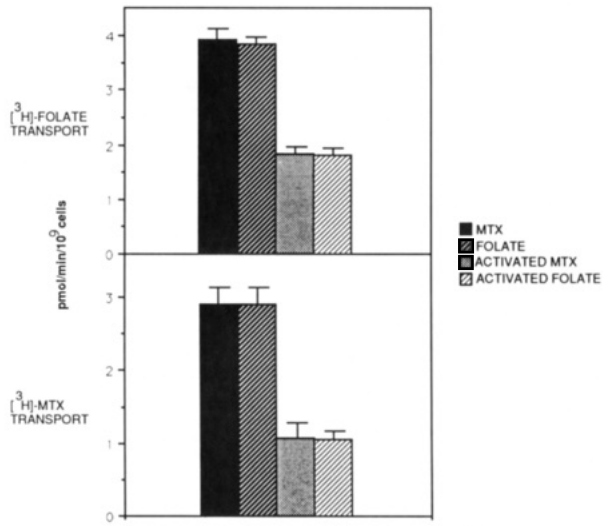


FIGURE 3: Irreversible inhibition of [$^3$H]folate and [$^3$H]methotrexate transport into wild-type *Leishmania* by EDC-activated ligands. The effects of EDC-activated folate and EDC-activated methotrexate on the ability of wild-type organisms to transport the corresponding nonactivated ligand are shown. DI700 cells were incubated under normal affinity labeling conditions with a 100-fold excess of either EDC-activated folate or EDC-activated methotrexate. Parallel incubations of DI700 cells in the presence of a 100-fold excess of nonactivated folate and methotrexate served as controls. The *Leishmania* were washed extensively in phosphate-buffered saline followed by centrifugation to remove any noncovalently bound material, and the cells were subjected to the standard transport assay conditions described under Experimental Procedures. The results depicted are the averages and standard deviations from three determinations.

determined after a 10-min incubation with "activated" folate or methotrexate. DI700 cells preincubated with identical concentrations of non-"activated" ligand served as controls. As shown by the data depicted in Figure 3, incubation of wild-type organisms with 40 $\mu$M "activated" folate or 40 $\mu$M "activated" methotrexate for 10 min decreased the ability of the cells to take up radiolabeled 0.1 $\mu$M folate by 50% and methotrexate by approximately 65%. These data suggest that 50–65% of the folate/methotrexate transporters were inactivated by 100-fold excess of the EDC-activated ligands. However, the possibility that 40 $\mu$M "activated" ligand interfered with transport in a nonspecific fashion cannot be eliminated.

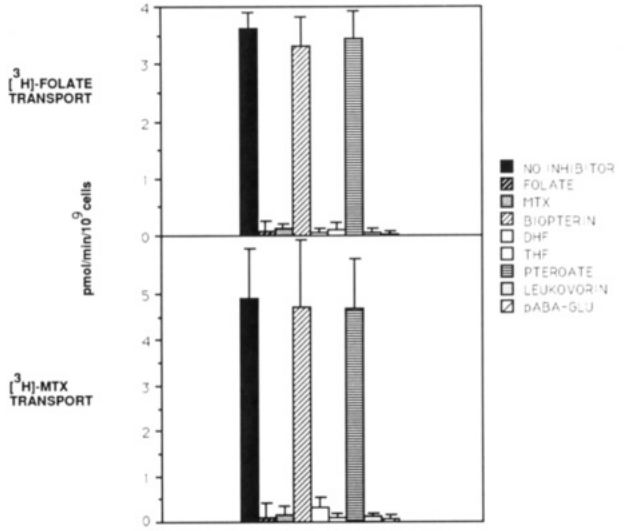


FIGURE 4: Effects of various pterins and folates on [$^3$H]folate and [$^3$H]methotrexate transport into wild-type organisms. The ability of wild-type cells to transport 0.4 $\mu$M [$^3$H]folate and 0.4 $\mu$M [$^3$H]-methotrexate in the absence and presence of a 100-fold excess concentration of one of a spectrum of pterin and folate derivatives is shown in the histogram. The results depicted are the means and standard deviations from at least three independent determinations.

*Effects of Competitive Ligands on Affinity Labeling.* To assess the effects of competitive ligands on affinity labeling of the 46 000-dalton protein, wild-type *Leishmania donovani* were incubated with 0.4 $\mu$M "activated" ligand in the presence of a 100-fold excess of a nonradiolabeled compound. The data indicate that those compounds that inhibited the translocation of either folate or methotrexate (Figure 4) from the culture medium into wild-type cells blocked the covalent labeling of the 46 000-dalton polypeptide by EDC-activated folate (Figure 5A) or EDC-activated methotrexate (Figure 5B). A similar 100-fold excess of folate, methotrexate, dihydrofolate, tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and (*p*-aminobenzoyl)glutamate blocked the influx of both folate and methotrexate (Figure 4) and inhibited completely the covalent adduct formation between the "activated" pteridines and the polypeptide (Figure 5). Equivalent concentrations of nonradioactive biopterin, pteroic acid, adenosine, and inosine, which did not interfere with the ability of wild-type cells to take up either radiolabeled ligand, did not inhibit the extent of affinity labeling by either "activated" ligand (Figures 4 and 5).

## Discussion

Membrane proteins have proven refractory to further structural analysis by virtue of the fact that they lose function after removal from the membrane milieu. Thus, the reconstitution of extracted proteins into artificial liposomes, a problematic and inconvenient procedure, has provided the only means of assaying for these proteins. A method by which functional proteins can be identified is by direct radiolabeling of the binding sites with analogues of substrates and inhibitors containing reactive or photoreactive groups that form covalent adducts. These affinity and photoaffinity labeling techniques have been particularly exploited to identify enzymes in crude extracts or in intact cells, and a variety of membrane-associated transport proteins have been identified in this fashion. However, the success of these labeling techniques is dependent on the availability or synthesis of reactive ligands. In these studies, we have exploited a protocol established by Henderson and colleagues which has been used previously to inactivate

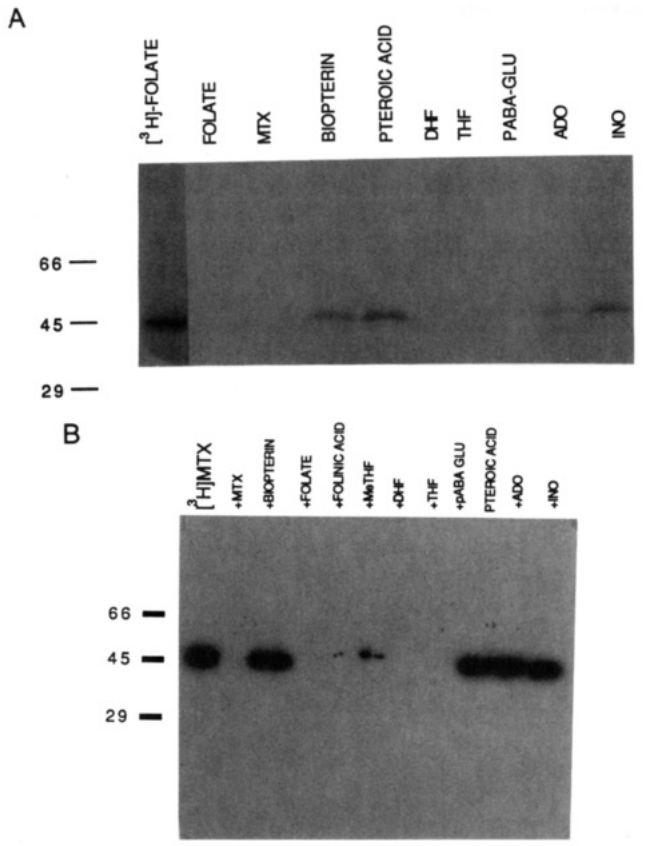


FIGURE 5: Effects of various pterins and folates on the affinity labeling of the 46 000-dalton polypeptide of DI700 cells by EDC-activated ligands. The abilities of the pterins and folates depicted in Figure 4 to block the labeling of the 46 000-dalton polypeptide by EDC-activated ligands are depicted. Cells were preincubated with a 100-fold excess (40 $\mu$M) of the nonradioactive folates and pterins and subjected to the normal affinity labeling procedure with either EDC-activated [$^3$H]folate (panel A) or [$^3$H]methotrexate (panel B) as described under Experimental Procedures. The products were analyzed by SDS gel electrophoresis.

(Henderson et al., 1980) the methotrexate carrier of mouse L1210 cells and to inactivate (Henderson & Potuznik, 1982) and label (Ananthanarayanan et al., 1984) the folate binding protein component in *Lactobacillus casei* to affinity label the folate/methotrexate carrier of *Leishmania donovani*, a pathogenic hemoflagellate that is the causative agent of visceral leishmaniasis. This affinity labeling technique has numerous advantages over many comparable protein labeling procedures including its simplicity, its specificity, and its inexpensiveness. Moreover, unlike many photolabeling methods, peptide bonds of the protein are not destroyed as a consequence of the affinity labeling.

Two species of *Leishmania*, *Leishmania major* and *Leishmania donovani*, have been shown to express a single common transport system for folate and methotrexate. Both genetic evidence and kinetic evidence support this contention (Ellenberger & Beverley, 1987a,b; Kaur et al., 1988). Ellenberger and Beverley (1987b) and Kaur et al. (1988) have isolated mutant strains of *Leishmania major* and *Leishmania donovani*, respectively, that are deficient in their abilities to transport both pteridines. The MTXA5 strain employed in these studies was isolated in a single step, suggesting, although not proving, that the ability to transport both ligands was a consequence of a single genetic lesion. That a single mutation caused the loss of both folate and methotrexate transport capability in the MTXA5 cells is further supported by the recent isolation of revertants of MTXA5 cells that have regained, either partially or totally, both folate and methotrexate transport capabilities (Beck and Ullman, unpublished data). Furthermore, competition studies with wild-type *Leishmania donovani* indicate that an excess of one ligand could inhibit the translocation of the other across the plasma membrane (Figure 4). A thorough and detailed kinetic analysis of folate and methotrexate transport into *Leishmania* has been performed (Ellenberger & Beverley, 1987a).

The MTXA5 cell line has proven invaluable in demonstrating that the 46 000-dalton polypeptide that is affinity labeled in wild-type organisms by both radiolabeled "activated" folate and radiolabeled "activated" methotrexate is associated with the transport of the unmodified pteridine ligands, since no covalent adduct is formed with a 46 000-dalton polypeptide or with any other protein when MTXA5 cells were incubated with 0.4 $\mu$M EDC-activated ligand. Kinetic evidence supported the hypothesis that the 46 000-dalton affinity-labeled polypeptide in wild-type *Leishmania* is associated with transport, since a plethora of competitive ligands of folate/methotrexate transport inhibited the formation of a covalent adduct with the 46 000-dalton protein. The substrate specificity of the folate/methotrexate carrier of *Leishmania donovani* is essentially the same as that ascribed to the same transporter in *Leishmania major* (Ellenberger & Beverley, 1987a).

Two sets of data indicated that the "activated" pteridine employed in these experiments interacted directly with the folate/methotrexate carrier on the cell surface of wild-type *Leishmania donovani*. First, incubation of wild-type DI700 cells with exogenous "activated" folate or "activated" methotrexate led to an irreversible inhibition of folate or methotrexate transport capability. Second, to eliminate the possibility that the 46 000-dalton polypeptide in wild-type cells is an intracellular protein which had been labeled differentially in DI700 and MTXA5 cells by virtue of the ability of the former to transport "activated" as well as unmodified ligands, membrane and cytosolic fractions were prepared from both cell lines. The data supported the premise that the affinity-labeled 46 000-dalton polypeptide in DI700 *Leishmania* is either the folate/methotrexate carrier or a binding protein directly associated with the folate/methotrexate carrier. A covalent adduct to the 46 000-dalton protein was found only after DI700 membrane preparations had been labeled with "activated" ligand, regardless of whether the membrane isolation step occurred before or after the EDC-directed labeling procedure. No labeled protein was observed in cytosolic subcellular fractions of either DI700 or MTXA5 cells. The latter observation was somewhat surprising since the leishmanial dihydrofolate reductase–thymidylate synthase (DHFR–TS) complex, a protein of higher molecular weight than the folate/methotrexate carrier (Garrett et al., 1984), is a methotrexate binding cytosolic protein. Perhaps EDC-activated methotrexate did not recognize the DHFR from *Leishmania donovani*, or it may not be reactive under the buffer or temperature conditions which are optimal for the labeling of the folate/methotrexate transporter. Alternatively, as the autoradiogram depicted in Figure 2 was developed after a relatively short exposure (1 week), it is possible that the low amounts of DHFR–TS present in wild-type *Leishmania* (Coderre et al., 1983; Kaur et al., 1988) could account for the apparent failure of the "activated" ligands to label the DHFR–TS in wild-type *Leishmania donovani* in these experiments. Later studies in this laboratory using *Leishmania major* that overproduce DHFR–TS (Ellenberger & Beverley, 1987b) have revealed that the affinity labeling technique can be exploited to covalently "tag" the leishmanial DHFR–TS

polypeptide, as well (data not shown). Using a similar procedure, Price et al. (1987) have demonstrated that $^{125}$I-labeled *N*-(4-amino-4-deoxy-10-methylpteroyl)-*N*-(4-azido-5-iodosalicylyl)-L-lysine (APA-ASA-Lys), a photoaffinity analogue of methotrexate, can be used to covalently label the DHFR protein in L1210 cells.

It is noteworthy that the 46 000 molecular weight assigned to the leishmanial folate–methotrexate transporter is virtually identical with that obtained for the folate binding protein associated with membrane transport in L1210 cells (Price & Freisheim, 1987; Price et al., 1988). Iodinated APA-ASA-Lys, the photoactivatable methotrexate analogue, forms a stable covalent bond with a 46 000–48 000-dalton polypeptide present in L1210 membranes as determined by SDS gel electrophoresis. This same group indicated that APA-ASA-Lys also reacts with a 38 000-dalton cytosolic peptide, which was hypothesized to participate in the shuttling of reduced folates to cytosolic targets (Price et al., 1988). The molecular weight assigned to the leishmanial and L1210 folate carriers differs substantially from those reported for folate binding proteins from other organisms. For instance, the folate binding protein purified from *Lactobacillus casei* has an apparent molecular weight of 25 000 (Henderson et al., 1977). In mammalian cells and tissues, an enormous disparity has been observed in the molecular weights of particulate or membrane-bound folate binding proteins. Molecular weights of 16 000–24 000 and 100 000 (Leslie & Rowe, 1972), 340 000–400 000 (Spector, 1977), 38 500 (Antony et al., 1981), and 28 000 and 310 000 (Sadasivan et al., 1986) have been reported for folate binding proteins isolated from rat brush border intestinal epithelial cells, rabbit choroid plexus, human placenta, and human leukemic cells, respectively.

The exact chemical identities of the "activated" species remain unknown. Henderson et al. have postulated that the reactive species of the EDC-modified folate or methotrexate might be an internal $\alpha,\gamma$-anhydride or, alternatively, a mono- or disubstituted urea derivative (Henderson et al., 1980; Kurzer & Douraghi-Zadeh, 1967). Regardless, the specific radiolabeling of the folate/methotrexate carrier of *Leishmania donovani* by the EDC-mediated affinity labeling technique described in these studies provides an avenue by which this transporter, and perhaps other functional membrane proteins, can be isolated by conventional protein purification techniques. Finally, the protocol described herein may be applicable to the isolation of other membrane transporters. Recently, we have developed the technology to affinity label two other membrane transporters in *Leishmania donovani*. *Leishmania* possess two genetically and biochemically distinguishable transporters for nucleosides, one for adenosine and pyrimidine nucleosides and one for inosine and guanosine (Iovannisci et al., 1984; Aronow et al., 1987). Using EDC-activated adenosine and EDC-activated inosine, covalent adducts have been formed with wild-type cells but not with cells genetically deficient in the corresponding specific nucleoside transport system.

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# Evidence for Functionally Distinct Glucose Transporters in Basal and Insulin-Stimulated Adipocytes†

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ABSTRACT: The activity and $K_m$ of glucose transport of rat adipocytes are quite variable in the basal state. This could be due to differing levels of highly saturable transport against a background of less saturable transport. Such heterogeneity could lead to differing conclusions as to the $K_m$ of basal cells compared to insulin-stimulated cells depending on the choice of substrate, the range of concentrations tested, and the rigor of data analysis. In the present work, we used a cell preparation which was stable and partially activated by constant agitation. We used a two-component model to fit the concentration dependence of D-glucose uptake. We defined two parallel pathways of glucose entry, a high-affinity/low-capacity pathway and a low-affinity/high-capacity pathway. Both pathways were stereospecific and were inhibited by cytochalasin B. The low-affinity pathway in basal cells had 97% of the total capacity ($V_{max}$) with a high $K_m$ (>50 mM). A second pathway had a very low $K_m$ (<1 mM) and only 3% of the total capacity, but contributed to 30–60% of glucose uptake at 8 mM glucose. In insulin-stimulated cells, a pathway with a $K_m$ of 4–5 mM dominated and contributed 85% of glucose transport. The low-affinity but not the very high affinity pathway persisted in stimulated cells, but its contribution was only 10–15% of transport at 8 mM glucose. These results suggest the presence of at least two functionally distinct transporters whose respective contributions can be characterized by nonlinear regression of data over a wide range of glucose concentrations. From the data, we conclude that an increase of the apparent affinity of glucose transport is important to insulin action and that this observation is consistent with the hypothesis that insulin recruits a functionally distinct type of transporter into the plasma membrane. These results are discussed in relation to recent immunological findings that several species of glucose transporters may coexist in the adipocyte membrane.

It has been generally accepted (Vinten & Gliemann, 1976; Olefsky, 1978; Whitesell & Gliemann, 1979) that insulin stimualtes glucose transport in adipocytes and other cells by increasing the $V_{max}$ without altering the $K_m$ of the process. This is consistent with the hypothesis that insulin acts primarily by mobilizing additional carriers to the plasma membrane which are functionally identical with those present in the basal state (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). We have presented evidence that, under certain conditions, rat adipocytes transport glucose with a high $K_m$ in the basal state and that this $K_m$ can be lowered by 90% in response to environmental factors, including insulin treatment (Whitesell & Abumrad, 1985, 1986). We suggested as alternatives either that insulin activated the basal transporters or that it recruited a nonconstitutive type of glucose carrier with a higher affinity for glucose. Discriminating between these two possibilities became a significant consideration, since multiple forms of the glucose carrier have been recently identified on the basis of structural homology and immunological evidence (Kayano et al., 1988). Similar probes also identified a distinct insulin-sensitive transporter in adipocytes and muscle. This transporter was not detected in plasma membranes of resting cells which express, however, a constitutive transporter (James et al., 1987). A glucose transporter active in liver membranes transports glucose with 90% lower affinity than does the erythrocyte transporter (Axelrod & Pilch, 1983), and this may be the liver glucose transporter recently cloned by Thorens et al. (1988). These studies, which show the coexistence of more than one type of transporter in a particular tissue, would imply functional heterogeneity of transport. This heterogeneity could apply to transport $K_m$ or substrate specificity, or both.

Okuno and Gliemann (1987), in an attempt to explain our finding that the $K_m$ of transport was changed by insulin in the adipocyte, suggested that "basal" preparations of adipocytes may in fact be heterogeneous to varying degrees, with glucose transported simultaneously by saturable and "non-saturable" processes. They suggested that our cell preparation may have been relatively homogenous for the nonsaturable process, which is not the physiologically important pathway for glucose entry. However, they did not characterize the nonsaturable component for uptake of glucose itself as regards stereospecificity and cytochalasin B sensitivity. The choice of substrate and its inhibitability are important because of the possibility of specificity differences among the facilitative transport path-

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