Photoaffinity Analogues of Methotrexate as Folate Antagonist Binding Probes. 2. Transport Studies, Photoaffinity Labeling, and Identification of the Membrane Carrier Protein for Methotrexate from Murine L1210 Cells†

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ABSTRACT: A membrane-derived component of the methotrexate/one-carbon-reduced folate transport system in murine L1210 cells has been identified by using a photoaffinity analogue of methotrexate. The compound, a radioiodinated 4-azidosalicylyl derivative of the lysine analogue of methotrexate, is transported into murine L1210 cells in a temperature-dependent, sulfhydryl reagent inhibitable manner with a K_t of 506 ± 79 nM and a V_{max} of 17.9 ± 4.2 pmol min^{-1} (mg of total cellular protein)$^{-1}$. Uptake of the iodinated compound at 200 nM is inhibited by low amounts of methotrexate (I_{50} = 1.0 μM). The parent compounds of the iodinated photoprobe inhibit [^{3}H]methotrexate uptake, with the uniodinated 4-azidosalicylyl derivative exhibiting a K_i of 66 ± 21 nM. UV irradiation, at 4 °C, of a cell suspension that had been incubated with the probe results in the covalent modification of a 46K–48K protein. This can be demonstrated when the plasma membranes from the labeled cells are analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. Labeling of this protein occurs half-maximally at a reagent concentration that correlates with the K_t for transport of the iodinated compound. Protection against labeling of this protein by increasing amounts of methotrexate parallels the concentration dependence of inhibition of photoprobe uptake by methotrexate. In addition, no labeling occurs when a cell line that has a defective methotrexate transport system is similarly treated. Evidence that, in the absence of irradiation and at 37 °C, the iodinated probe is actually internalized is demonstrated by the labeling of two soluble proteins (M_r 38K and 21K) derived from the cell homogenate supernatant.

The transport of methotrexate (MTX)[1] into L1210 murine leukemia cells occurs via a carrier-mediated, saturable, energy- and temperature-dependent process [reviewed in Goldman (1971), Huennekens et al. (1978), and Sirotnak (1980)]. This internalization occurs via a high-affinity transport system for 5-methyltetrahydrofolate (5-MeFAH$_4$),[1] and a recent report indicates that folate is also transported by this system (Henderson et al., 1986). The efficacy of MTX as a chemotherapeutic agent is dependent on the ability of the target cell to accumulate the drug to a level that will more than saturate the target enzyme dihydrofolate reductase (DHFR).[1] It is this excess of MTX that ensures efficient cytotoxicity (Sirotnak, 1980). Occasionally, target cells become resistant to MTX, whereby the cells develop a mechanism that overcomes the cytotoxicity of the compound. Accordingly, cells that cannot accumulate MTX due to a defective transport system have been described (McCormick et al., 1981). While the transport of MTX into L1210 cells has been extensively studied, there is a paucity of information regarding the molecular characteristics of the membrane-bound protein responsible for the binding and internalization of MTX.

The ability of L1210 cells to bind, but not transport, MTX at 4 °C has facilitated the detection and partial characterization of the binding macromolecule expressed on the surface of these cells (Henderson et al., 1980b). This component demonstrates a high affinity for both 5-MeFAH$_4$, the physiologically predominant folate, and MTX. The carrier exhibits K_d values for 5-MeFAH$_4$ and MTX that correlated closely with the K_t values for the transport of these compounds. In addition, since an excess of unlabeled MTX can displace bound ^{14}C-labeled 5-MeFAH$_4$ at 4 °C, it is concluded that internalization does not occur at this temperature. Studies involving the identification and isolation of the carrier have proven difficult due to the low levels of the transport protein expressed on the plasma membranes of L1210 cells.

Inhibition of folate and MTX transport into L1210 cells can be achieved with a number of different reagents. These include compounds structurally dissimilar to folates such as sulfhydryl reagents (Rader et al., 1974; Henderson & Zevely, 1981), 8-azidoadenosine 5′-monophosphate (Henderson et al., 1979), and 4,4′-diisothiocyanostilbene-2,2′-disulfonate (Henderson & Zevely, 1982). Carbodiimide-activated folates (Henderson et al., 1980a) and the *N*-hydroxysuccinimidyl ester of MTX (NHS-MTX)[1] (Henderson & Montague-Wilkie, 1983) have been shown to be competent inhibitors of MTX transport in L1210 cells. Of all the above compounds, NHS-MTX was the most potent inhibitor of MTX transport (I_{50} = 20 nM).

The technique of photoaffinity labeling offers a specific method for covalent modification of macromolecules. Photoaffinity reagents are ligands that contain a reactive nitrene moiety that is activated by UV irradiation. This approach

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[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate (4-amino-4-deoxy-10-methylfolic acid); 5-MeFAH$_4$, 5-methyltetrahydrofolate; NHS-MTX, *N*-hydroxysuccinimidyl methotrexate; APA-[^{125}I]ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[^{125}I]iodosalicylyl)-L-lysine; APA-ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azidosalicylyl)-L-lysine; pCMS, *p*-(chloromercuri)benzenesulfonate; APA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-L-lysine; TLC, thin-layer chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPO, 2,5-diphenyloxazole; $(CH_3)_2$POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene; Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

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allows the study of reversible, competitive interactions between the label and the target macromolecule, be it in intact cells or as a purified preparation, when the experiment is performed in subdued light, in the absence of irradiation. Once irradiated, the photoaffinity ligand will covalently modify the protein to which it is bound (Cavalla & Neff, 1985). The compound 2-azidoaminopterin has been synthesized and utilized as a photoaffinity label for bacterial DHFR (Holmes et al., 1982), but the labeling efficiency was quite low.

Our laboratory recently reported the synthesis of two series of photoaffinity analogues of MTX (Price et al., 1986b). These are the 4-azidosalicylyl derivatives of the lysine and ornithine analogues of MTX. One of the compounds, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[^{125}I]-iodosalicylyl)-L-lysine (APA-[^{125}I]ASA-Lys),[1] has been utilized to covalently modify L1210 DHFR in a specific, UV irradiation dependent reaction (Price et al., 1987). The photoaffinity labeling was both specific and reasonably efficient, indicating the usefulness of the analogue as a photoaffinity reagent for the modification of folate binding proteins.

This paper describes the application of photoaffinity labeling to the identification of the putative MTX/reduced folate carrier protein that resides in the plasma membranes of L1210 cells. Both APA-ASA-Lys[1] and APA-[^{127}I]ASA-Lys[1] proved to be potent competitive inhibitors of MTX transport into L1210 cells at 37 °C. The effectiveness of the latter compound in transport inhibition led to the use of APA-[^{125}I]ASA-Lys in the photoaffinity labeling of the carrier protein. Data to substantiate the identification of the labeled protein as a component of the methotrexate transport system in L1210 cells are also presented. A preliminary account of this work has been reported elsewhere (Price et al., 1986a).

Experimental Procedures

Materials. RPMI 1640 powdered media was obtained from Irvine Scientific, Irvine, CA. Penicillin and streptomycin were obtained from KC Biologicals, Lenexa, KS. Supplemented calf serum was purchased from HyClone Laboratories, Logan, UT. Methotrexate was a generous gift from Dr. John A. R. Mead, Division of Cancer Treatment, National Cancer Institute, NIH. [3,5,9-^{3}H]Methotrexate (20 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA, and was purified via cellulose TLC (Eastman Kodak) immediately prior to use, with 0.2 M ammonium acetate as the mobile phase. Carrier-free ^{125}I (as Na^{125}I in dilute NaOH) was obtained from Amersham. Silica TLC plates, with fluorescent indicator, were obtained from The Anspec Co., Ann Arbor, MI. Gel electrophoresis reagents were from Bio-Rad, Rockville Centre, NY. All other supplies and reagents were of the highest quality commercially available.

Cell Culture. L1210/S (MTX-sensitive) and L1210/R81 cells, an MTX-resistant, transport-defective subline (McCormick et al., 1981), were grown in RPMI 1640 media supplemented with 5% calf serum and 100 units of penicillin and 100 μg of streptomycin per mL. L1210/R81 cells were grown in the presence of 10 μM MTX as described (McCormick et al., 1981). The L1210/R81 line was grown in MTX-free media for at least 20 doublings prior to its use in photoaffinity labeling experiments. Viability of the cells was evaluated by Trypan blue exclusion. In no case, either before or after experimental manipulation, was the viability less than 95%.

Transport Measurements. Cells were harvested at a density of 1.0–1.5 × 10^6 cells/mL by centrifugation (2600*g*, 5 min). The pellet was washed once with 160 mM HEPES and 2 mM $MgCl_2$, pH 7.4 (buffer A), and resuspended in buffer A at a density of 2 × 10^7 cells/mL. For uptake determinations, the cell suspension was equilibrated at 37 °C for 5 min. Following the addition of the radiolabeled compound, duplicate 1-mL aliquots were removed and mixed with 5 mL of 0.9% ice-cold NaCl. The cells were pelleted by a 90-s spin in an IEC clinical centrifuge, setting 7. The pellets were quickly washed twice in ice-cold saline (5 mL each), and the final pellet was dried by inversion for 2 h and solubilized by the addition of 600 μL of Soluene (Packard Instruments, Downers Grove, IL). The cell-associated radioactivity was determined by mixing the solubilized cell solution with 10 mL of scintillation cocktail [5 g of PPO, 300 mg of $(CH_3)_2$POPOP/1 L of toluene] and counting the samples in a Beckman LS3145P scintillation counter. The above procedure was repeated with cells that had been equilibrated at 4 °C. Results are expressed as picomoles of compound per milligram of protein (10^7 cells = 1.3 mg). Inhibition constants were obtained by determining the initial rate of [^{3}H]MTX uptake at various [^{3}H]MTX concentrations as a function of inhibitor concentration. Measurements were made at 30, 60, 90, and 120 s. The rate of [^{3}H]MTX uptake was linear during these time intervals. Dixon analysis of the data gave the K_i values. Lineweaver–Burk analysis of the initial linear rate of APA-[^{125}I]ASA-Lys uptake as a function of concentration yielded the K_t and V_{max} values. Measurements were made at 15, 30, 45, and 60 s. APA-[^{125}I]ASA-Lys uptake was linear during this time period. In either case, the plots were obtained by least-squares linear regression analysis, where the r (correlation) value was at least 0.95. All manipulations involving the photoaffinity analogues were performed in subdued light.

Synthesis of APA-[^{125}I]ASA-Lys. The radiolabeled probe was synthesized as previously described (Price et al., 1986b). Approximately 50 μg of APA-ASA-Lys was dissolved in 50 μL of dry dimethylformamide. ^{125}I (1 mCi) (as a 10-μL aliquot in dilute NaOH) was added, followed by the addition of two Iodobeads (Pierce Chemical Co., Rockford, IL). After 2 min, the solution was applied to a 5 × 20 cm silica TLC plate and developed in 3:1:1 2-propanol/methanol/NH_4OH. The product was visualized by autoradiography and short-wave UV fluorescence quenching. The product-containing silica gel was scraped into a tube and extracted into 1 mL of methanol. The silica gel was pelleted by a 5-min spin in a minifuge (Fisher), and the methanol was aspirated and dried under a stream of nitrogen. The product was dissolved in buffer A and stored at −70 °C. The concentration of the derivative was determined spectrophotometrically by absorbance at 302 nm and at pH 13, with a molar extinction coefficient of 22 100 M^{-1}. When a specific amount of APA-ASA-Lys, determined by the above spectrophotometric method, was subjected to amino acid analysis, the expected content of lysine was found. The product was stable for ca. 7 days, after which time multiple radioactive spots were detected when the compound's purity was determined by TLC and autoradiography.

L1210 Cell Labeling. Cells were harvested and washed as described above. The cells were resuspended in buffer A at a density of 1 × 10^8 cells/mL, and 1 mL of this suspension was placed in one well of a 24-well polystyrene cell culture dish (Costar, Cambridge, MA). The dish was placed in an ice–water bath and the cell suspension equilibrated at 4 °C for 5 min. The subsequent steps were performed in subdued light. To the suspension was added the appropriate concentration of APA-[^{125}I]ASA-Lys, and the suspension was mixed via trituration, incubated for 1 min, and then irradiated (15 mW/cm^2; Spectroline Model B-100, Spectronics Corp., Westbury, NY) with long-wave UV irradiation for 30 s. The solution was immediately made 5% (v/v) in β-mercapto-

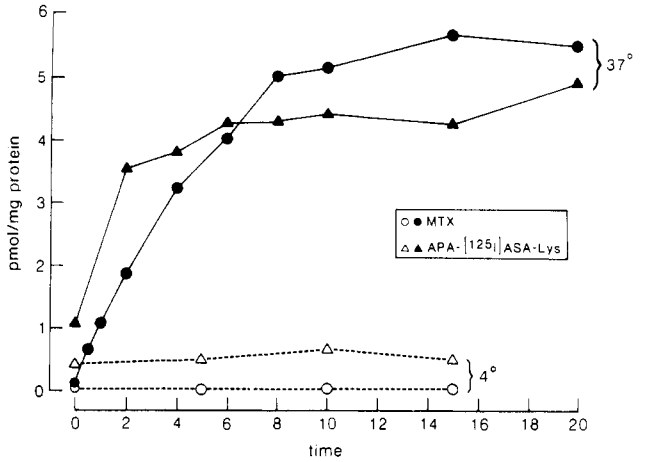

FIGURE 1: Uptake of APA-[^{125}I]ASA-Lys and [^{3}H]MTX into L1210/S cells. Uptake was assayed as described under Experimental Procedures. L1210/S cells (2×10^7 cells/mL in buffer A) were equilibrated at either 37 or 4 °C, in subdued light, for 5 min. The cell suspension was made 200 nM in iodinated photoprobe or [^{3}H]-MTX, and duplicate 1-mL aliquots were removed at the indicated times (minutes) and processed as described. The data points reflect the average of duplicate time points from two separate experiments.

ethanol, and plasma membranes were prepared as described previously (Henderson & Zevely, 1984). Prior to electrophoresis, when indicated, the membranes were washed with 0.3% saponin once and with buffer A three times.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Plasma membranes (50 μg), determined according to the method of Bradford (1976) or Kalb and Bernlohr (1977), were solubilized in 62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 140 mM dithiothreitol, and 6 M urea by incubating the samples at 60 °C for 2 h. After a brief centrifugation to remove insoluble material, the samples were loaded onto the slab gel and electrophoresis was performed with 30 mA of constant current. The gels were stained with Coomassie Blue R-250, dried, and exposed to Kodak X-Omat film for 5–14 days with intensifying screens (Du Pont). The molecular weight standards were a premixed kit from Bio-Rad. They are myosin H chain (200K), phosphorylase *b* (97.4K), bovine serum albumin (68K), ovalbumin (43K), and α-chymotrypsinogen (25.7K).

RESULTS

Transport of APA-[^{125}I]ASA-Lys. As shown in Figure 1, APA-[^{125}I]ASA-Lys (200 nM) is rapidly transported into L1210/S cells. This uptake is clearly temperature dependent, as there is essentially no accumulation of the compound at 4 °C. Uptake of [^{3}H]MTX at 37 °C, at an extracellular concentration of 200 nM, is shown, and the results indicate that MTX and APA-[^{125}I]ASA-Lys are both concentrated by L1210/S cells to the extent of approximately 5 pmol/mg of protein in a temperature-dependent fashion.

Figure 2 shows the effect of the sulfhydryl reagent *p*-(chloromercuri)benzenesulfonate (pCMS)[1] on APA-[^{125}I]-ASA-Lys transport. After 2 min of 37 °C uptake, at a pCMS concentration of 10 μM, uptake of the iodinated photoaffinity analogue (200 nM) is reduced by 50%. This correlates well with the effect of pCMS on the uptake of [^{3}H]MTX (Rader et al., 1974; Henderson & Zevely, 1981); 10 μM pCMS, added to the cell suspension simultaneously with [^{3}H]MTX, inhibits uptake of the folate antagonist by 50%. Inhibition of APA-[^{125}I]ASA-Lys uptake by unlabeled MTX was examined, and the results are indicated in Figure 3. Uptake of 200 nM photoprobe was measured at a 2-min time point in the presence of increasing concentrations of MTX added to the cell suspension simultaneously with the iodinated compound. Uptake of APA-[^{125}I]ASA-Lys was reduced by 50% by a concentration of MTX of 1.0 μM.

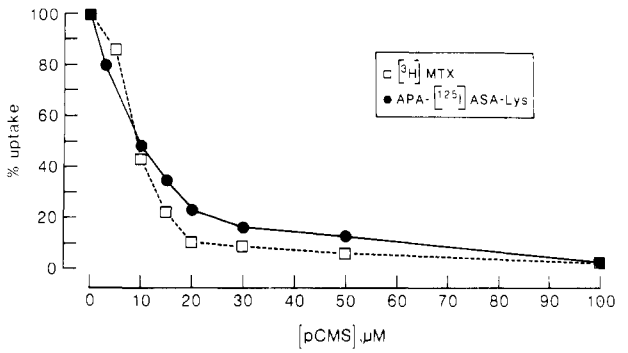

FIGURE 2: Inhibition of APA-[^{125}I]ASA-Lys and [^{3}H]MTX uptake by pCMS. L1210/S cells (2×10^7 cells/mL) were incubated at 37 °C for 5 min in subdued light. Immediately after the addition of the appropriate concentration of pCMS, the suspension was made 200 nM in APA-[^{125}I]ASA-Lys or [^{3}H]MTX. After 2 min, duplicate 1-mL aliquots were removed and processed as described. Results are presented as a percent of control, which was a 2-min uptake in the absence of pCMS. Data points are the average of duplicate data points from two separate experiments.

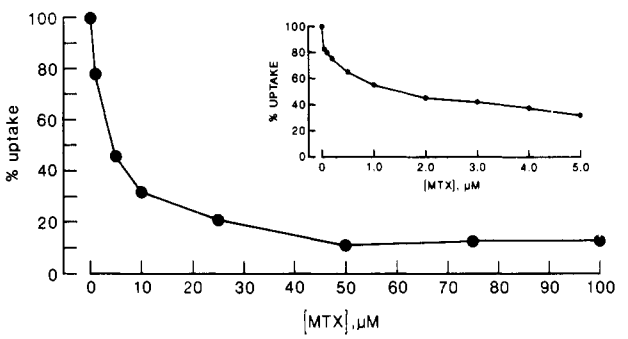

FIGURE 3: Inhibition of APA-[^{125}I]ASA-Lys uptake into L1210/S cells by MTX. Uptake was measured as described in the legend for Figure 2, where nonradioactive MTX was used as the inhibitor. Data points are the average of duplicate data points from three separate experiments on different days. Values did not vary by more than 10%. The inset indicates the percent uptake inhibition by MTX in the 0–5.0 μM concentration range.

Table I: Transport Kinetics of the Interaction between Analogues of MTX and L1210/S Cells

compound	K_i (nM)[a]	K_t (nM)[b]	V_{max}[b] [pmol min^{-1} (mg of protein)$^{-1}$]
APA-Lys	10300 ± 7500		
APA-ASA-Lys	66 ± 21		
APA-[^{127}I]ASA-Lys	8.0 ± 0.7		
APA-[^{125}I]ASA-Lys		506 ± 79	17.9 ± 4.2
[^{3}H]MTX		2800	14.8

[a] The K_i values were obtained from Dixon plots where the initial rate of transport of two different concentrations of [^{3}H]MTX was determined as a function of increasing amounts of inhibitor. [b] The K_t and V_{max} values were obtained from Lineweaver–Burk plots.

Table I describes the results of the kinetic analysis of the reversible interaction between the photoaffinity analogues and the MTX transport system in L1210/S cells. These experiments were performed under subdued light. The K_t value was obtained from a Lineweaver–Burk plot, and the K_i values were derived from a Dixon plot of the data. The K_t for APA-[^{125}I]ASA-Lys was determined to be 506 ± 79 nM, a value approximately 5.5-fold lower than the K_t for MTX (2800 nM). The V_{max} was 17.9 ± 4.2 pmol min^{-1} (mg of protein)$^{-1}$, a value quite similar to the V_{max} value [14.8 pmol min^{-1} (mg of protein)$^{-1}$] for [^{3}H]MTX uptake. The kinetic constants for

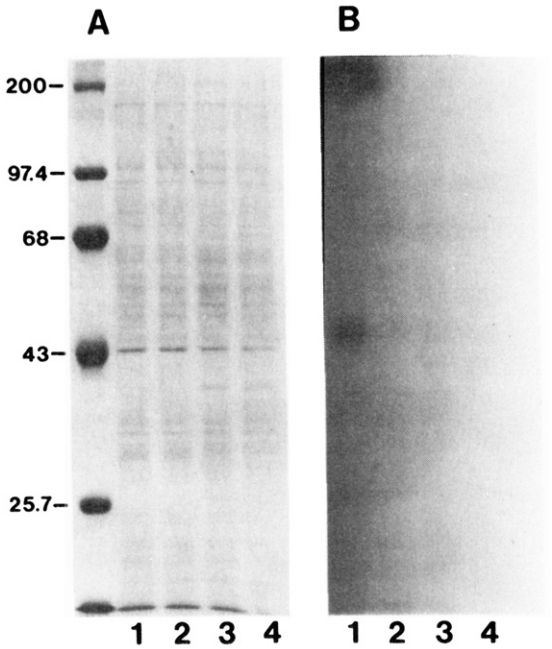

FIGURE 4: Photoaffinity labeling of L1210/S and L1210/R81 cells. L1210 cells (1×10^8) in 1 mL of buffer A at 4 °C were labeled with 200 nM APA-[^{125}I]ASA-Lys as described under Experimental Procedures. Plasma membranes from each type of labeled cells were prepared, and 50 μg from each was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After being stained with Coomassie Blue R-250, the gel was dried and exposed to X-ray film, with intensifying screens, for 14 days. (Panel A) Coomassie-stained gel: (lane 1) labeled membranes from L1210/S cells; (lane 2) labeled membranes from L1210/S cells that had been preincubated with 200 μM MTX; (lane 3) labeled membranes from L1210/R81 cells; (lane 4) labeled membranes from L1210/R81 cells that had been preincubated with 200 μM MTX. Molecular weight standards are described under Experimental Procedures. (Panel B) Autoradiogram obtained from the dried gel. The lanes correspond to those in panel A (1–4).

[^{3}H]MTX uptake obtained in our laboratory agree well with the previously reported values of 14 pmol min^{-1} (mg of protein)$^{-1}$ for the V_{max} and 900–4500 nM, depending on the type of HEPES buffer used for the K_t determinations (Henderson & Zevely, 1980). APA-Lys,[1] the parent compound of the photoaffinity analogue used in this study, inhibited [^{3}H]MTX uptake with a K_i value of 10 300 ± 7500 nM while APA-ASA-Lys had a K_i of 66 ± 21 nM, a value that approaches the K_i (20 nM) for the inhibitor NHS-MTX (Henderson & Montague-Wilkie, 1983). In addition, the nonradioactive iodinated compound has a K_i value of 8.0 ± 0.7 nM.

Photoaffinity Labeling of the MTX Carrier Protein on L1210 Cells. Figure 4 shows the results of a photoaffinity labeling experiment in which both L1210/S and L1210/R81 transport-defective cells were studied. The latter cell line is resistant to MTX by virtue of an inability of the cell to transport the drug (McCormick et al., 1981). Cells were labeled as described under Experimental Procedures, and 50 μg of plasma membranes was solubilized directly in Laemmli protein sample buffer, which contained 140 mM dithiothreitol, 6 M urea, and 2% sodium dodecyl sulfate. Panel A of Figure 4 is the Coomassie Blue stained gel, and panel B is the autoradiogram of the same gel following electrophoresis. The photoaffinity labeling procedure clearly identifies a protein from L1210/S cells that has a M_r of approximately 46K–48K (Figure 4B, lane 1). When a 1000-fold molar excess of MTX is preincubated with the cells at 4 °C for 5 min, labeling of this protein is abolished (Figure 4B, lane 2). When the labeling experiment was repeated with transport-defective L1210/R81 cells, under conditions identical with those described for L1210/S cells, no labeling of a 46K–48K protein is observed (Figure 4B, lanes 3 and 4). In fact, the only protein labeled by this procedure using L1210/R81 cells is albumin, which can be visualized as a faint band (M_r 68K) in all lanes (Figure 4B). Longer exposure of the gel to film allows better visualization of the labeled albumin. Immunoprecipitation using anti-bovine serum albumin IgG (Sigma) was used to identify this protein as albumin, and when the labeling experiment was performed on an aliquot of cell-free serum-supplemented RPMI 1640, albumin was heavily labeled by APA-[^{125}I]ASA-Lys (data not shown).

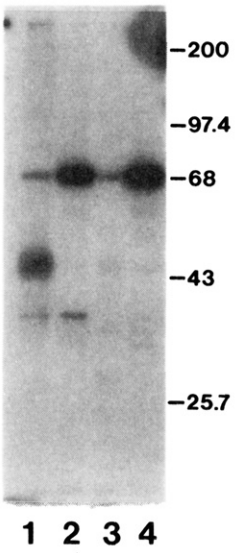

FIGURE 5: Photoaffinity labeling of whole L1210/S cells at 37 °C. Cells, equilibrated at 37 °C for 5 min, were incubated with 200 nM APA-[^{125}I]ASA-Lys for an additional 5 min. Following 30 s of irradiation, plasma membranes were prepared and both the cell homogenate supernatant (50 μg of protein) and plasma membranes (50 μg of protein) were analyzed with a 10% gel. Shown is the autoradiogram obtained from the dried gel. (Lane 1) Labeled plasma membranes; (lane 2) the supernatant from a 47000g centrifugation of a labeled cell homogenate; (lane 3) labeled plasma membranes from cells preincubated with 200 μM MTX. (lane 4) the supernatant from the sample described for lane 3 preincubated with 200 μM MTX.

When the sodium dodecyl sulfate–polyacrylamide gel of plasma membranes derived from labeled cells was exposed to X-ray film for an extended period of time, a faintly labeled protein (M_r 38K) can be visualized (data not shown). When the 45000g cell homogenate supernatant from the plasma membrane preparation is analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography, in addition to a heavily labeled band corresponding to albumin, a faintly labeled protein of M_r 38K is also observed. Figure 5 shows the autoradiogram obtained from a photoaffinity labeling experiment performed at 37 °C rather than at 4 °C. The labeling procedure is essentially the same as that described at 4 °C, with the exception that a 5-min incubation of the cells with the iodinated probe at 37 °C prior to a 30-s UV irradiation period was done. Lane 1 of Figure 5 is the labeling pattern observed for the plasma membranes from the cells labeled at 37 °C, and lane 2 is the labeling pattern observed in the cell homogenate supernatant from the preparation of the plasma membranes from these same cells. As indicated in the figure, at 37 °C, the 38K protein is 10-fold more heavily labeled in the homogenate supernatant fraction than in the plasma membrane fraction; this is based on scanning densitometry. (At 4 °C, labeling of this 38K protein in the supernatant fraction is ca. 85% less than that at 37 °C.) Additionally, no labeling of a 46K–48K protein is observed in the supernatant fraction. When the cells are preincubated for 5

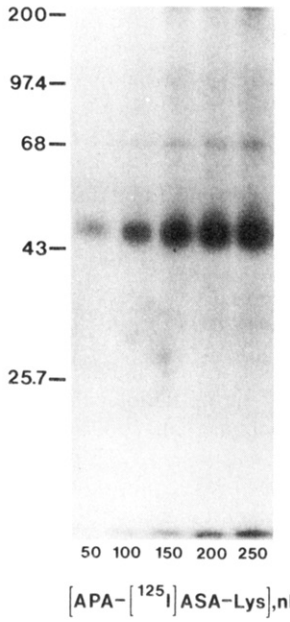

FIGURE 6: Concentration dependence of labeling of L1210/S cells with APA-[^{125}I]ASA-Lys. Cells (1×10^8) in 1 mL of buffer A were labeled as described under Experimental Procedures with the indicated concentrations of radioiodinated photoprobe. Following the preparation of plasma membranes, 50 μg of each preparation was washed with 0.3% saponin, followed by buffer A. The washed membranes were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography. Shown is the film following 10 days of exposure.

min at 37 °C with a 1000-fold molar excess of MTX prior to incubation with the probe and irradiation, no labeling of either the 46K–48K plasma membrane derived protein or the 38K soluble protein is observed (lanes 3 and 4). This autoradiogram also indicates that albumin is present to a greater extent in the soluble fraction (lanes 2 and 4) than in the plasma membrane fraction (lanes 1 and 3). Furthermore, when the cells are incubated for a longer period of time with a higher concentration of APA-[^{125}I]ASA-Lys prior to irradiation, a labeled 21K protein is also observed in the cell homogenate supernatant fraction, corresponding to cytosolic DHFR (data not shown).

The concentration of APA-[^{125}I]ASA-Lys required to label the membrane carrier half-maximally was determined by labeling L1210/S cells at 4 °C with increasing concentrations of the photoprobe. The concentrations used ranged from 50 to 600 nM, a range that brackets the K_t for 37 °C uptake of the iodinated photoaffinity analogue (cf. Table I). Figure 6 depicts the results of these experiments. L1210/S cells were labeled as described with the indicated amount of APA-[^{125}I]ASA-Lys. Prior to electrophoresis, the plasma membranes derived from the labeled cells were washed with 0.3% saponin as described under Experimental Procedures. This procedure removed the trace amounts of the contaminating 38K soluble protein from the plasma membrane preparation. Scanning densitometry of the autoradiogram revealed that half-maximal labeling of the carrier protein is achieved at a photoprobe concentration of approximately 150 nM. The intensity of the radiolabeled 46K–48K band remained constant at iodinated probe concentrations greater than 250 nM under these conditions (Figure 6).

Similarly, the concentration of MTX required to protect the carrier protein from labeling was determined. Concentrations of MTX ranged from 50 to 5000 nM, and the results of one such experiment are shown in Figure 7. The autoradiogram in Figure 7 shows the results of this experiment when L1210/S

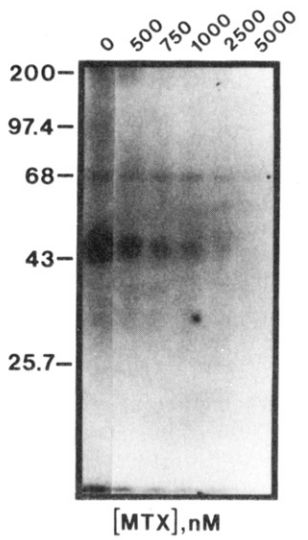

FIGURE 7: Concentration dependence of protection of L1210/S cells from photoprobe labeling with increasing amounts of MTX. Cells were labeled with 200 nM APA-[^{125}I]ASA-Lys as described (Figure 6) in the presence of the indicated amount of MTX, which was added simultaneously with the photoprobe. Plasma membranes were processed as described in Figure 6. Each lane contained 50 μg of protein. The autoradiogram following 10 days of exposure of the film is indicated.

cells are labeled at 4 °C with 200 nM APA-[^{125}I]ASA-Lys in the presence of increasing concentrations of MTX. Cells were labeled as described in Figure 6 except that the indicated amount of MTX was added with the iodinated probe to the cell suspension. Scanning densitometry indicated that the concentration of MTX which half-maximally protects labeling of the carrier protein was ca. 500 nM.

Discussion

Sources in which folate-binding proteins play a possible role in folate acccumulation and have been detected and isolated include *Lactobacillus casei* (Henderson et al., 1977), porcine choroid plexus (Suleiman & Spector, 1981), human placenta (Antony et al., 1981), and human KB cells (Antony et al., 1985; Kanee et al., 1986). Studies involving the isolation of the plasma membrane derived carrier protein in murine L1210 cells (Henderson et al., 1980b) have been hampered both by the paucity of carrier molecules present on these cells and by the lack of a suitably sensitive means of following the carrier protein through various analytical and preparative procedures. By use of the irreversible transport inhibitor *N*-hydroxysuccinimidyl [^{3}H]MTX, a 36K protein was visualized after autoradiography of a sodium dodecyl sulfate–polyacrylamide gel of a Sephacryl S-300 purified Triton extract of plasma membranes derived from whole cells labeled at 25 °C (Henderson & Zevely, 1984). Incorporation of a 50000-fold molar excess of MTX during the labeling of cells reduced significantly the amount of radioactivity recovered from the Sephacryl S-300 step. There are a number of disadvantages surrounding the use of this particular derivative of MTX in the labeling of the carrier protein. First, descriptions of the synthesis of the compound (Henderson & Montague-Wilkie, 1983; Henderson & Zevely, 1984) give no indication of the chemical or physical characterization of the NHS ester of MTX that would verify the identity and structure of the product. Furthermore, the product was not separated from unreacted starting material, which could include nonesterified MTX if the reaction did not proceed to completion, and *N*-hydroxysuccinimide and EDC, of which a 5000-fold excess of each was used. Second, the labeling experiment was per-

formed at 25 °C, a temperature at which there could be internalization of the compound provided that reaction between the nucleophilic imido ester of the MTX derivative and any primary amines or thiol groups in the binding pocket of the carrier protein is not rapid. Nonesterified MTX would also be internalized, as would any NHS-MTX that had been hydrolyzed during the incubation period. Any intracellular proteins labeled in such a fashion would be "pseudospecifically" labeled, as an excess of MTX (vide supra) would prevent the internalization of the derivative. Also, the amount of Triton X-100 required for half-maximal solubilization of the 36K protein (0.04%, 0.64 mM on the basis of a M_r of 628 for Triton X-100) is less than is expected to be required for the solubilization of an integral membrane protein (Kirkpatrick et al., 1974). Although these arguments generate doubt as to the conclusion that the 36K protein labeled by NHS-[^{3}H]MTX is an integral membrane-derived component of the MTX transport system, it is felt that, as this compound is a potent and specific inhibitor of [^{3}H]MTX transport (Henderson & Montague-Wilkie, 1983; Henderson & Zevely, 1984), the putative membrane-associated carrier might indeed have been covalently modified by the derivative, but in an inefficient manner that would render the tritium-labeled protein undetectable. The results described herein suggest that this 36K protein is either cytosolic or, perhaps, loosely associated with the plasma membrane.

This study describes the application of photoaffinity labeling to identify a component of the MTX transport system in L1210 cells. This same approach has been used to specifically modify L1210 dihydrofolate reductase in a UV irradiation dependent fashion (Price et al., 1987). The iodinated photoaffinity analogue of MTX, APA-[^{125}I]ASA-Lys, when irradiated with long-wave UV light in the presence of whole L1210/S cells at 4 °C, covalently modifies a single protein found in the plasma membranes of these cells. There is a good deal of evidence that indicates that this protein is a functional component of the MTX transport system in L1210 cells. The kinetic values reported in Table I show that APA-[^{125}I]ASA-Lys is efficiently transported into L1210/S cells in a manner similar to that for MTX. The V_{max} value for uptake of the radioiodinated probe is similar to that for MTX; however, the K_t for the photoprobe is 5.5-fold lower than the K_t for [^{3}H]-MTX uptake into L1210/S cells. In addition, Figures 1 and 2 indicate that both [^{3}H]MTX and the iodinated photoprobe, at the same extracellular concentration, are accumulated to the same extent in a temperature-dependent manner and that this uptake is pCMS inhibitable. Furthermore, low concentrations of MTX effectively inhibit uptake of the probe (Figure 3). These results indicate that APA-[^{125}I]ASA-Lys is transported into L1210/S cells via the same carrier-mediated transport system as that utilized by MTX and should, therefore, be a useful compound for the covalent modification of the MTX carrier protein. The temperature dependence of labeling of a 38K soluble protein (Figure 5) and a 21K soluble protein (not shown) indicates that the probe actually enters cells and does not simply bind to membrane components of the cell. This finding also corroborates the conclusion that the 36K protein identified via the labeling, at 25 °C, of whole cells with NHS-[^{3}H]MTX is actually a cytosolic or loosely associated peripheral membrane folate-binding protein. A soluble protein of very similar size (38K) is only faintly labeled when cells are irradiated in the presence of the photoprobe at 4 °C. However, when the cells are labeled at 37 °C (Figure 5), much heavier labeling of this 38K soluble protein is observed. Additionally, this protein copurified with the plasma membranes prepared from labeled cells, and a mild detergent (0.3% saponin) treatment effectively removed the 38K component but not the 46K–48K component.

Of some interest is the observation of labeled albumin (Figures 4–7), of which only a small fraction actually copurifies with plasma membranes from labeled L1210 cells. This is, apparently, the first demonstration that albumin, derived from the serum-supplemented media in which the cells are grown, is associated with the plasma membranes of these cells. As albumin binds MTX (Goldman, 1971; Liegler et al., 1969; Steele et al., 1979) biphasically and with relatively low affinities [71×10^4 M^{-1} and 0.18×10^4 M^{-1} (Steele et al., 1979)], it probably contributes significantly to the high degree of nonspecific binding of radiolabeled folate analogues to L1210 cells (Henderson et al., 1980b). Salicylate also binds to albumin (Geddes & White, 1979; Mais et al., 1974), although there is some discrepancy in the reported association constants, which range from 8.6×10^3 M^{-1} (Mais et al., 1974) to about 5.6×10^5 M^{-1} (Geddes & White, 1979). Since the reactive moiety of the photoprobe is a salicylate derivative (*p*-azidosalicylate), it is not unexpected that an excess of unlabeled MTX will not totally block labeling of albumin because labeling probably occurs at both the MTX and salicylate binding sites.

A 46K–48K protein is identified when whole cells are labeled with APA-[^{125}I]ASA-Lys at 4 °C. This protein is derived from the plasma membrane and is visualized when these plasma membranes are analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography (Figure 4). Additional evidence supporting the identification of this protein as participating in MTX transport is corroborated by the total absence of labeling of a 46K–48K protein in a cell line (L1210/R81) that shows a complete lack in ability to transport MTX (Figure 4). Furthermore, the amount of probe required to label the 46K–48K protein half-maximally at 4 °C (150 nM; Figure 6) is of the same order of magnitude as the K_t for transport of the photoprobe at 37 °C (506 ± 79 nM; Table I). The amount of MTX necessary to block labeling of the 46K–48K protein (500 nM; Figure 7) with 200 nM iodinated probe is similar to the concentration of MTX required to inhibit the 37 °C uptake of 200 nM probe (Figure 3). Labeling of the 46K–48K protein with 200 nM APA-[^{125}I]ASA-Lys was completely blocked in the presence of 5 μM 5-methyltetrahydrofolate (data not shown). The 46K–48K integral membrane carrier protein and the 38K soluble binding protein may be structurally related since both proteins bind MTX and related analogues. Whether there is a precursor–product relationship between these two proteins or the involvement of a common precursor is currently under investigation.

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Registry No. MTX, 59-05-2; APA-[^{125}I]ASA-Lys, 108919-01-3; APA-ASA-Lys, 107089-46-3; APA-Lys, 80407-56-3; folic acid, 59-30-3.

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Yeast Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate α,β-Phosphorylase Behaves as a Dinucleoside Tetraphosphate Synthetase

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ABSTRACT: The diadenosine 5′,5‴-P^1,P^4-tetraphosphate α,β-phosphorylase (Ap_4A phosphorylase), recently observed in yeast [Guranowski, A., & Blanquet, S. (1985) *J. Biol. Chem. 260*, 3542–3547], is shown to be capable of catalyzing the synthesis of Ap_4A from ATP + ADP, i.e., the reverse reaction of the phosphorolysis of Ap_4A. The synthesis of Ap_4A markedly depends on the presence of a divalent cation (Ca^{2+}, Mn^{2+}, or Mg^{2+}). In vitro, the equilibrium constant $K = ([Ap_4A][P_i])/([ATP][ADP])$ is very sensitive to pH. Ap_4A synthesis is favored at low pH, in agreement with the consumption of one to two protons when ATP + ADP are converted into Ap_4A and phosphate. Optimal activity is found at pH 5.9. At pH 7.0 and in the presence of Ca^{2+}, the V_m for Ap_4A synthesis is 7.4 s^{-1} (37 °C). Ap_4A phosphorylase is, therefore, a valuable candidate for the production of Ap_4A in vivo. Ap_4A phosphorylase is also capable of producing various Np_4N' molecules from NTP and N′DP. The NTP site is specific for purine ribonucleotides (N = A, G), whereas the N′DP site has a broader specificity (N′ = A, C, G, U, dA). This finding suggests that the Gp_4N' nucleotides, as well as the Ap_4N' ones, could occur in yeast cells.

Dinucleoside oligophosphates have been evidenced in numerous organisms [Rapaport & Zamecnik, 1976; Ogilvie & Jakob, 1983; Lüthje & Ogilvie, 1983; Garrison & Barnes, 1984; Morioka & Shimada, 1984; McLennan & Prescott, 1984; Garrison et al., 1986; Segal & Le Pecq, 1986; Baltzinger et al., 1986; reviewed in Silverman and Atherly (1979), Zamecnik (1983), and Grummt (1983)]. Among them, diadenosine tetraphosphate (Ap_4A) has been reported to vary

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