Characterization of the Methotrexate Transport Pathway in Murine L1210 Leukemia Cells: Involvement of a Membrane Receptor and a Cytosolic Protein†

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ABSTRACT: A radioiodinated photoaffinity analogue of methotrexate, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azidosalicylyl)-L-lysine (APA-ASA-Lys), was recently used to identify the plasma membrane derived binding protein involved in the transport of this folate antagonist into murine L1210 cells [Price, E. M., & Freisheim, J. H. (1987) *Biochemistry 26*, 4757–4763]. The labeled protein has an apparent molecular weight of 46K–48K when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, but no such labeling occurs in a methotrexate transport-defective cell line (L1210/R81). Labeling of the total cytosolic protein from disrupted cells, followed by electrophoresis and autoradiography, showed, among other proteins, a 21K band, corresponding to dihydrofolate reductase (DHFR), in both the parent and R81 cells and a 38K band only in the parent cells. However, when whole cells were UV irradiated at various times at 37 °C following addition of radiolabeled APA-ASA-Lys, the 38K protein and DHFR were the only cytosolic proteins labeled in the parent cells, while the intact R81 cells showed no labeled cytosolic protein, since the photoprobe is not transported. Further, when the parent cells were treated with a pulse of radiolabeled photoprobe, followed by UV irradiation at different times at 37 °C, the probe appeared sequentially on the 48K membrane protein and both the 38K cytosolic protein and dihydrofolate reductase. The protoprobe eventually dissociated from the 38K protein and was found associated with other intracellular proteins at 10–12 min, corresponding to the time required for methotrexate uptake to reach a steady-state level. A 48K protein could be detected in both parent L1210 cells and the R81 cells on Western blots using antisera to a membrane folate binding protein from human placenta. However, the uptake of radiolabeled APA-ASA-Lys by L1210 cells was inhibited by methotrexate but not by folate, suggesting the binding/transport proteins for folate and reduced folate coenzymes are separate but may be immunologically related. These results suggest a vectorial transport of APA-ASA-Lys or methotrexate and reduced folate coenzymes into murine L1210 cells mediated by a 48K integral membrane protein and a 38K cytosolic or peripheral membrane protein. The 38K protein may help in the trafficking of reduced folate coenzymes, shuttling them to various cytosolic targets.

The chemotherapeutic agent methotrexate (MTX),[1] a 2,4-diamino-10-methyl analogue of the vitamin folic acid, is one of the most commonly used anticancer agents. The cytotoxic effectiveness of MTX is related to the ability of the target cell to accumulate the drug (Kessel et al., 1965). Furthermore, resistance to MTX is often manifested by a decrease in the transport of the drug from the extracellular space to the interior of the cell [reviewed in Albrecht and Biedler (1984)]. MTX is transported into drug-sensitive murine L1210 cells via an energy- and temperature-dependent, saturable, carrier-mediated process [reviewed in Goldman (1971), Dembo and Sirotnak (1984), and Henderson (1986)]. This transport system is shared by other reduced folates such as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate (Henderson et al., 1986). Although the major intracellular target for MTX, dihydrofolate reductase (DHFR), has been relatively well characterized from a host of sources [reviewed in Freisheim and Matthews (1984)], proteins involved in MTX transport have only recently been identified.

One of the first cases in which a purified folate binding protein was implicated in playing a role in folate transport was in studies with *Lactobacillus casei* (Henderson et al., 1977). The purified carrier protein from this source had an apparent molecular weight of 20K–25K and exhibited binding properties similar to those observed in the intact transport system. Recently, a membrane-derived folate binding protein was purified from human nasopharyngeal epidermoid carcinoma cells (KB cells), and this protein had an apparent molecular weight of 50K (Elwood et al., 1986). This protein cross-reacted with antisera raised against a folate binding protein purified from human placenta (Antony et al., 1985), and the antisera blocked folate influx into KB cells (Kane et al., 1986). These results indicate that the 50K membrane-derived protein is involved in folate transport into KB cells.

When a radioiodinated photoaffinity analogue of MTX, APA-[^{125}I]ASA-Lys, was utilized, a 46K–48K membrane-derived protein was identified on murine L1210 cells and was

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[1] Abbreviations: MTX, methotrexate [N^{α}-(4-amino-4-deoxy-10-methylpteroyl)glutamic acid]; DHFR, dihydrofolate reductase (EC 1.5.1.3); APA-ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azidosalicylyl)-L-lysine; APA-[^{125}I]ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[^{125}I]iodosalicylyl)-L-lysine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; "folate" is used to mean the ionized form of folic acid as opposed to its reduced coenzyme forms.

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determined to be the plasma membrane bound MTX carrier protein from these cells (Price & Freisheim, 1987). This protein is distinguished from the membrane folate transport proteins seen in other mammalian cells in that it is more specific for the reduced folate coenzymes such as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate and methotrexate rather than folate (Henderson et al., 1986). The photoprobe used in this work has also been used to covalently modify purified L1210 DHFR in a specific fashion (Price et al., 1987). Furthermore, an *N*-hydroxysuccinimide ester of MTX has been used to identify a 36K protein from L1210 cells which was also suggested to be a component of the MTX transport system in these cells (Henderson & Zevely, 1984).

The data presented herein suggest the involvement of a temporal series of binding events which describe MTX transport into murine L1210 cells and distinguish it from the folate transport pathway. The individual proteins which participate in this transport system were identified by the techniques of photoaffinity labeling and immunoblotting. A working model is also presented which provides a biochemical link between the 46K–48K membrane-derived binding protein and a soluble 38K protein.

Materials and Methods

A radioiodinated photoaffinity analogue of MTX, APA-[^{125}I]ASA-Lys, was synthesized as previously described (Price & Freisheim, 1987). The MTX-sensitive L1210/S cell line and the MTX-resistant L1210/R81 cell line were grown as previously described (Price & Freisheim, 1987). The latter cell line is resistant to MTX due to both an overproduction of DHFR (35-fold) and a marked inability to transport the drug (McCormick et al., 1981). MTX was a generous gift from Dr. John A. R. Mead, Division of Cancer Treatment, National Cancer Institute, NIH, and was purified by high-performance liquid chromatography prior to use.

Uptake of APA-[^{125}I]ASA-Lys. Cells growing in log phase were washed once with 160 mM HEPES/2 mM $MgCl_2$, pH 7.5, and resuspended in the same buffer at a density of 2×10^7 cells/mL. The cells were preincubated for 4 min at either 37 °C or 0 °C, and duplicate 1-mL aliquots were incubated with 800 nM APA-[^{125}I]ASA-Lys alone or together with various concentrations of MTX or folate for a further 4 min. The cells were then transferred to ice after adding 5 mL of ice-cold NaCl solution (0.9%), pelleted at 3000*g* for 3 min, washed twice with the same solution, and counted for radioactivity. These operations were carried out under a red light. The total cpm of the cells at 4 °C were subtracted from the corresponding values at 37 °C to obtain the final values.

Photoaffinity Labeling Studies. Intracellular proteins were labeled with APA-[^{125}I]ASA-Lys either in intact cells at 37 °C or as an aliquot of homogenized cells from which plasma membranes were removed by centrifugation (43000*g* for 5 min). For intact cells, L1210/S or L1210/R81 cells (1×10^8 cells/mL in 160 mM HEPES/2 mM $MgCl_2$, pH 7.4, adjusted with 1 N NaOH; "buffer A") were equilibrated at 37 °C for 5 min. All of the following steps were performed in very subdued light. The cell suspension was made 500 nM in APA-[^{125}I]ASA-Lys, and 1-mL aliquots were removed after 0, 5, and 10 min of incubation at 37 °C. The aliquot was placed in 1 well of a 24-well culture dish that was floating in an ice/water bath and irradiated for 60 s with long-wave UV light (15 mW/cm^2, Spectroline Model B-100, Spectronics Corp.). The irradiated cells were made 5% (v/v) in β-mercaptoethanol, admixed with 5 mL of ice-cold buffer A, and pelleted by a 90-s centrifugation in an IEC clinical centrifuge, setting 7. The labeled cells were washed twice more with ice-cold buffer A, and plasma membranes and cell homogenate supernatants were prepared as described below. In one instance, unlabeled MTX (50 μM) was added simultaneously with the photoprobe to the cell suspension, and the cells were irradiated after 10 min of influx at 37 °C. A control experiment was performed as above except the cells were maintained at 4 °C throughout the course of the experiment. The proteins in the cell homogenate supernatants were precipitated by the addition of acetone to a final concentration of 50% (v/v). After 3 h at −20 °C, the precipitated proteins were recovered by centrifugation and were analyzed via SDS–PAGE and autoradiography.

The second method of labeling intracellular proteins from either L1210/S cells or L1210/R81 cells involved the disruption of unlabeled cells (2 mL, 1×10^8 cells/mL in buffer A) by Dounce homogenization followed by high-speed centrifugation. An aliquot of the supernatant from this centrifugation, corresponding to 10 μg of protein (0.5-mL total volume), was incubated with 200 nM APA-[^{125}I]ASA-Lys in subdued light for 5 min in 1 well of a 24-well cell culture dish that was floating in ice/water. The solution was then irradiated for 60 s and made 50% (v/v) in acetone. After 3 h at −20 °C, the precipitated proteins were analyzed by SDS–PAGE and autoradiography. A parallel experiment was performed which involved preincubation of the sample with 50 μM MTX for 5 min prior to the addition of the radioiodinated photoprobe.

Finally, a "pulse" labeling experiment was performed utilizing intact L1210/S cells. Cells (12 mL; 1×10^8 cells/mL in buffer A) were equilibrated at 4 °C for 5 min. The suspension was then made 500 nM in APA-[^{125}I]ASA-Lys and incubated an additional 5 min in subdued light at 4 °C. The cells were then pelleted and resuspended in 12 mL of photoprobe-free buffer A that had been preequilibrated at 37 °C. After the cells had been resuspended in this buffer (ca 15 s), a 1-mL aliquot was removed, placed in 1 well of a 24-well culture dish that was floating in ice/water, and irradiated for 30 s. The labeled cells were made 5% (v/v) in β-mercaptoethanol and admixed with 5 mL of ice-cold buffer A. After the labeled cells were pelleted, they were washed twice more, and both a purified plasma membrane fraction and a cell homogenate supernatant fraction were prepared. The remainder of the cells were maintained at 37 °C, and additional 1-mL samples were removed every 2 min (up to and including 20 min) and processed as just described. The plasma membrane fractions and cell homogenate supernatant fractions from each time point were analyzed via SDS–PAGE and autoradiography.

Plasma Membrane Preparation. Plasma membranes were purified from labeled cells according to the method of Koizumi et al. (1981) as modified by Henderson and Zevely (1984). Briefly, the cells were exposed to an ice-cold hypotonic buffer containing 1.0 mM phenylmethanesulfonyl fluoride, 1.0 μM pepstatin A, 1.0 mM 1,10-phenanthroline, and 1.0 mM iodoacetamide and homogenized by 50 manual strokes in a tight-fitting Dounce glass homogenizer. Whole cells and intact nuclei were cleared from the homogenate by 3000*g* centrifugation for 5 min. The crude membranes were pelleted after 43000*g* centrifugation for 45 min. The supernatant from this centrifugation was termed the "cell homogenate supernatant". The plasma membranes in the pellet were further purified by centrifuging through a discontinuous sucrose gradient as described (Henderson & Zevely, 1984). The activity of the plasma membrane marker enzyme Na^+,K^+-ATPase in the partially purified plasma membrane preparation was enriched

about 33-fold, compared to the whole cell homogenate. Additionally, no Na^+,K^+-ATPase activity was detected in the cell homogenate supernatant fraction. The Na^+,K^+-ATPase assay was performed according to the method of Scharschmidt et al. (1979).

Gel Electrophoresis. SDS–PAGE was performed according to the method of Laemmli (1970). Protein concentrations were determined either by the method of Bradford (1976), using bovine serum albumin as the standard, or by the method of Kalb and Bernlohr (1977). In order to prepare plasma membrane proteins for electrophoresis, an appropriate volume of the membrane preparation was subjected to centrifugation (15 min in a Minifuge at 4 °C). The pellet was dissolved in 75 μL of Laemmli protein sample buffer containing 140 mM dithiothreitol and 6 M urea. Dissolution was achieved by heating the samples to 70 °C for 2–3 h. Proteins from cell homogenate supernatants were prepared for electrophoresis by either acetone or trichloroacetic acid precipitation. There were no noticeable differences in the labeling pattern obtained when the soluble proteins were precipitated by either method. The precipitated proteins were recovered by centrifugation and dissolved in the above Laemmli sample buffer. After the samples were completely solubilized, they were loaded onto a slab gel in an electrophoresis apparatus (Bio-Rad), and electrophoresis was performed at 30 mA until the dye front was ca. 0.5 cm from the bottom of the gel. The gel was stained with Coomassie R-250, destained, dried, and exposed to Kodak X-omat film. Intensifying screens (Du Pont) were always used.

Production of Antisera. The membrane folate binding protein from human placenta, purified essentially as described by Antony et al. (1981), was used as the immunogen. The protein (0.5 mg) was injected into a rabbit intradermally in Freund's complete adjuvant. Three weeks later, a booster dose of 100 μg of protein was injected intradermally in Freund's incomplete adjuvant followed by bleeding, a week later.

Immunoblots. Membrane vesicles from L1210 cells corresponding to 50 μg of total protein were solubilized in sample buffer, electrophoresed on SDS–polyacrylamide gels (15%), and blotted onto nitrocellulose. The blots were probed with rabbit antisera (1:1000) to human folate binding protein in 10 mM sodium phosphate (pH 7.5)/150 mM NaCl/5% nonfat dry milk and then with peroxidase-conjugated goat anti-rabbit IgG and developed in sodium acetate buffer containing H_2O_2 and 4-chloronaphthol as described (Ratnam et al., 1987).

Results and Discussion

When intact MTX-sensitive L1210/S cells were irradiated after incubation with APA-[^{125}I]ASA-Lys at the transport-permissive temperature of 37 °C, an intracellular 38K protein was heavily labeled in a time-dependent fashion (Figure 1, lanes 1–3). Two other intracellular proteins were similarly labeled; one had an apparent molecular weight of 34K, and the second had an apparent molecular weight of 21K and is presumably dihydrofolate reductase (DHFR). Also, as previously described (Price & Freisheim, 1987), albumin is slightly labeled by this procedure and can be visualized, upon longer exposure, as a faint band at 68K in all lanes. Inclusion of 50 μM MTX in the cell labeling reaction resulted in a marked decrease, but not an abolishment, in the labeling intensity of the 38K protein (lane 4). MTX almost completely blocked the labeling of DHFR in this experiment and essentially had no effect on the labeling pattern of the 34K protein. These data indicate that the 38K protein (as well as DHFR) is a specific MTX binding protein and the 34K protein is a nonspecifically labeled species. When the time course of labeling was repeated at 4 °C, only labeled albumin was detected

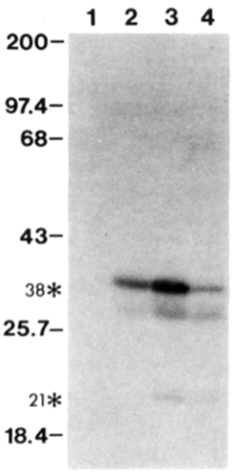

FIGURE 1: Photoaffinity labeling of intracellular proteins from intact L1210/S cells under transport-permissive conditions. A suspension of L1210/S cells (3.5 mL; 1×10^8 cells/mL) was equilibrated at 37 °C for 5 min. The following steps were performed in subuded light. The suspension was made 500 nM in APA-[^{125}I]ASA-Lys, and aliquots were removed at $t = 0$, 5, and 10 min and irradiated as described under Materials and Methods. A cell homogenate supernatant was obtained, and 75 μg of protein was acetone-precipitated (−20 °C, 3 h) and analyzed via SDS–PAGE and autoradiography. Shown is the autoradiogram obtained after an 8-day exposure. Lanes 1–3, labeling pattern observed after 0, 5, and 10 min, respectively, of incubation with the photoprobe at 37 °C. Lane 4, labeling pattern observed after 10 min of uptake at 37 °C in the presence of 50 μM MTX. Molecular weight standards (Bethesda Research Laboratories) were myosin H-chain (200K), phosphorylase B (97.4K), bovine serum albumin (68K), ovalbumin (43K), α-chymotrypsinogen (25.7K), and β-lactoglobulin (18.4K). The molecular weights marked by asterisks were calculated graphically by plotting the log of the molecular weight of the standards versus the distance which that standard migrated following electrophoresis.

in the cell homogenate supernatant (data not shown). These results indicate that APA-[^{125}I]ASA-Lys, at 37 °C, is transported into L1210/S cells and associates specifically with a 38K protein and DHFR. The experiment was also performed utilizing intact MTX-resistant L1210/R81 cells, and only labeled albumin was detected in the cell homogenate supernatant fraction prepared from cells labeled at either 37 °C or 4 °C (data not shown). These results are consistent with our previous findings that these cells are resistant to MTX through an inability to transport the drug (McCormick et al., 1981).

In order to directly identify a 38K MTX binding protein in the cytosol of L1210 cells, aliquots of the cell homogenate supernatants from both L1210/S and L1210/R81 cells were labeled. In addition, the specificity of the labeling reaction was examined by labeling aliquots of cell homogenate supernatants from both cell lines with the photoprobe in the presence of unlabeled MTX. The results of these experiments are presented in Figure 2. Lane 1 of this figure shows heavy labeling of a 38K protein from L1210/S cytosol, and lane 2 indicates that unlabeled MTX completely prevents this modification. This approach also nonspecifically labeled a number of other intracellular proteins, albeit much more faintly. Lane 3 of Figure 2 indicates that DHFR is heavily labeled in an aliquot of cell homogenate supernatant from L1210/R81 cells where the enzyme is 35-fold overproduced, but no labeling of a 38K protein is detected. Unlabeled MTX effectively prevented labeling of the L1210/R81 DHFR (lane 4). The complete absence of labeling of a 38K protein in cytosols from MTX transport-defective cells may implicate this protein as being involved in MTX uptake in drug-sensitive L1210/S cells. Comparison of the data in Figure 2 with those in Figure 1

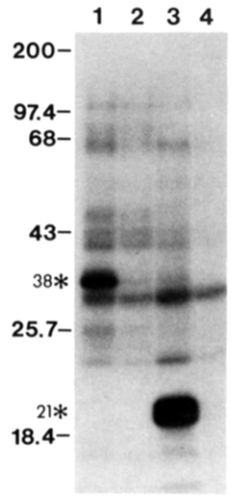

FIGURE 2: Photoaffinity labeling of intracellular proteins derived from homogenized, unlabeled L1210/S and L1210/R81 cells. Cells were homogenized as described under Materials and Methods, and an aliquot of the cell homogenate supernatant (corresponding to 10 μg of protein) was incubated at 4 °C with 200 nM APA-[^{125}I]ASA-Lys for 5 min. Following irradiation, the proteins were acetone-precipitated and analyzed by SDS–PAGE and autoradiography. Shown is the autoradiogram following an 8-day exposure. Also, an aliquot of the cell homogenate supernatant was preincubated with 50 μM MTX for 5 min prior to the additon of the photoprobe. Lane 1, labeling pattern observed in an aliquot of cell homogenate supernatant from L1210/S cells; lane 2, same as lane 1, except MTX was present; land 3, labeling pattern observed in an aliquot of cell homogenate supernatant from L1210/R81 cells; lane 4, same as lane 3, except MTX was present. Protein (10 μg) was added to each lane, and molecular weight standards were those described in the legend to Figure 1.

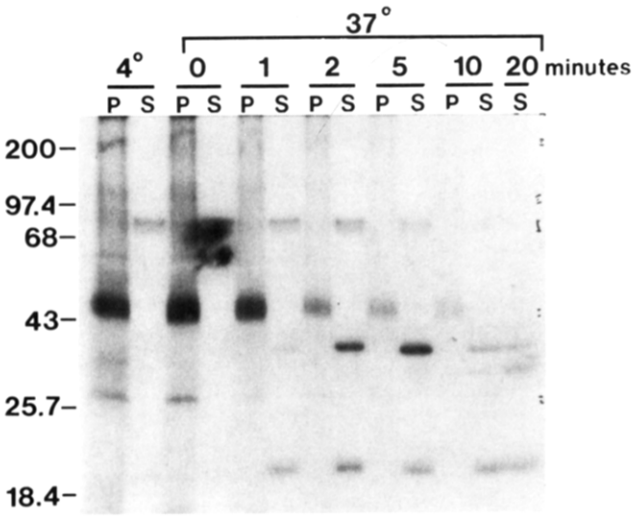

FIGURE 3: Pulse labeling of L1210/S cells. Cells were incubated in subdued light, with 500 nM APA-[^{125}I]ASA-Lys at 4 °C for 5 min. After the cells were washed with buffer A, they were incubated at 37 °C, as described under Materials and Methods. At the indicated times, a 1-mL aliquot was removed and irradiated. Both a purified plasma membrane fraction and a cell homogenate supernatant fraction were obtained, and each was analyzed via SDS–PAGE and autoradiography. Shown is the autoradiogram from an 18-day exposure. Lanes marked "P" are purified plasma membranes (30 μg), and lanes indicated by an "S" are aliquots (75 μg) of cell homogenate supernatant. Molecular weight standards are those described in the legend to Figure 1.

substantiates this interpretation. When intact, transport-competent L1210/S cells were radiolabeled with photoprobe in the presence of unlabeled MTX, the 38K protein was only partially protected. If the 38K protein were involved in MTX transport, then the MTX added as a protecting agent would be transiently associated with it. This would result in only *partial* protection (Figure 1, lane 4). However, if the 38K protein is uncoupled from the transport machinery of the L1210/S cell by homogenization, then any MTX added to the homogenate would remain bound to this species. This would result in complete protection of the 38K protein (Figure 2, lane 2). Furthermore, when disrupted, homogenized cells were subjected to photoaffinity labeling, a number of proteins were nonspecifically modified (Figure 2, lanes 1 and 2) compared to the single 34K protein that was nonspecifically labeled when whole cells were labeled at 37 °C (Figure 1, lane 4). These results may indicate that the probe (or MTX, by inference) does not diffuse freely into the cell following internalization but follows a specific pathway. Finally, as shown in Figure 1, DHFR is one of the few intracellular proteins labeled when whole L1210/S cells were irradiated in the presence of the photoprobe under transport-permissive conditions. However, under the conditions described in Figure 2 when disrupted cells were labeled, a number of intracellular proteins were labeled, while DHFR was faintly labeled at this time of exposure of the autoradiograph (Figure 2, lane 1). This result further suggests that in intact cells MTX transport follows a distinct route leading to preferential binding by a 38K protein and DHFR.

The experiment shown in Figure 3 indicates that the 38K intracellular protein identified in Figures 1 and 2 is a principal intracellular constituent of the MTX influx pathway. Indicated in the figure is the autoradiogram obtained from a time course of labeling experiment in which cells were "pulsed" with 500 nM APA-[^{125}I]ASA-Lys at 4 °C (a temperature where binding but not transport occurs), washed, and then incubated at the transport-permissive temperature of 37 °C (Figure 3). The only available photoprobe for transport is that which was initially bound at 4 °C on the surface of the cell. As indicated, the labeling intensity of the 46K–48K membrane carrier protein diminished as a function of time. After 10 min of uptake, essentially no labeling of this transporter is detected. Concomitant with the loss of label from the membrane carrier is the appearance of photoprobe associated with the 38K intracellular MTX binding protein (t = 2, 5 min). In addition, at later time points, after 10 min, the label is lost from the 38K protein as well and can be faintly visualized as being distributed to other intracellular proteins. The observed loss of label from the 38K protein at t = 10 min could have been due to simple efflux of the photoprobe from this species to the exterior of the cell. This possibility was ruled out because the amount of trichloroacetic acid precipitable radioactivity in the cell homogenate supernatant remained constant at times later than about 5 min (data not shown). This suggests that the loss of radioactivity from the 38K protein is actually due to a redistribution of the photoprobe to other intracellular proteins. Interestingly, the point at which the label is lost from the intracellular 38K protein (t = 10 min) correlates well with the time at which uptake of [^{3}H]MTX or APA-[^{125}I]ASA-Lys reaches a steady state (Price & Freisheim, 1987). These data further indicate that the 38K intracellular protein is involved in MTX uptake, during the initial period of active transport where transport is unidirectional.

We have observed that plasma membrane vesicles prepared from cells labeled with APA-[^{125}I]ASA-Lys show the presence of a 38K labeled protein which can be dissociated from the membranes by treatment with 0.3% saponin (Price & Freisheim, 1987). This observation suggests that the 38K protein could indeed be loosely associated with the cytoplasmic surface of the membrane, thus facilitating transfer of the probe from the 46K–48K membrane protein.

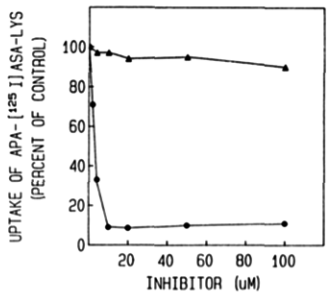

FIGURE 4: Specificity of the transport system for APA-[^{125}I]ASA-Lys for MTX as compared with folate. The uptake of the labeled probe by L1210/S cells in a 2-min period was measured in 160 mM HEPES/2 mM $MgCl_2$, pH 7.5 at 37 °C, at a probe concentration of 800 nM. The uptake was measured at various concentrations of folate (▲) or MTX (●) as described under Materials and Methods. The values are expressed as percent of a control that did not contain either folate or MTX. The cpm due to background, obtained for each point at 4 °C, was subtracted from the corresponding values at 37 °C.

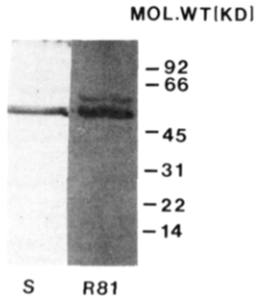

FIGURE 5: Western blots of plasma membrane protein (50 μg) from L1210/S (S) and L1210/R81 (R81) cells. The blots were probed with antibodies to the purified membrane folate receptor from human placenta (1:1000) and peroxidase-conjugated goat anti-rabbit IgG as described under Materials and Methods.

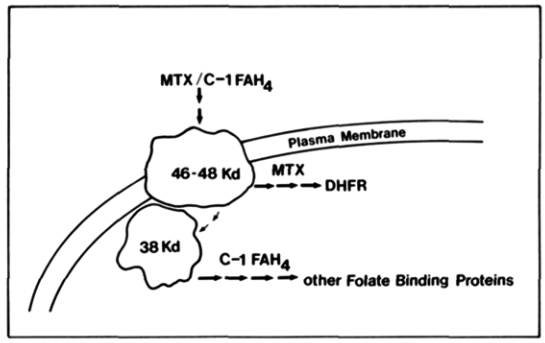

FIGURE 6: Proposed molecular model for the MTX/one-carbon tetrahydrofolate influx pathway in L1210/S cells.

Of the many known intracellular folate binding enzymes, only thymidylate synthase has a subunit molecular weight (M_r 34K) in the range of the 38K protein (Rode et al., 1979). It is unlikely that the 38K intracellular MTX transport component is thymidylate synthase for two reasons. First, the MTX-resistant cells (L1210/R81) and L1210 parent cells contain virtually equal amounts of thymidylate synthase, on the basis of 5′-fluoro[^{3}H]deoxyuridylate assays (data not shown). Furthermore, as shown in Figure 2, lane 3, no photoaffinity-labeled 38K protein can be detected in an aliquot of homogenized R81 cells. Second, the monoglutamate form of MTX is a poor inhibitor of thymidylate synthase and should therefore not prevent the labeling of this enzyme. As shown in Figure 2, lane 2, a 250-fold excess of MTX virtually completely protects the 38K protein from labeling.

Also shown in Figure 3 is labeled DHFR, which appears at early periods of influx. It should be pointed out that, unlike the labeling pattern of the 38K cytosolic protein, the labeling intensity of DHFR remains virtually constant at all times, even at later stages of influx (t = 20 min). This result indicates that a constant amount of photoprobe is associated with the reductase with a high affinity after a brief period of influx (t = 2 min). This correlated with the previous finding that the probe is a potent inhibitor of purified L1210 DHFR in the absence of irradiation (Price et al., 1986). The photoprobe complex of the 38K protein, on the other hand, must at later time points (>10 min) be subjected to conditions that cause dissociation of the probe.

In light of reports on the presence of separate membrane transport systems for MTX or reduced folate coenzymes and folate in mammalian cells, it was of interest to examine the specificity of the transport system for APA-[^{125}I]ASA-Lys for MTX. As indicated in Figure 4, while low concentrations of MTX (5 μM) dramatically inhibit influx of the photoprobe into L1210 cells, folate, even at relatively high concentrations (100 μM), did not significantly inhibit this influx. This result is consistent with a recent report on the relative affinities of folate and MTX for the reduced folate transport system in L1210 cell membranes (Henderson et al., 1986).

The current findings also brought forth data pertaining to the phenomenological events regarding MTX resistance due to a transport defect. Results from this laboratory have previously demonstrated that, although a 46K–48K protein from the plasma membranes of MTX transport-competent L1210/S cells was labeled with APA-[^{125}I]ASA-Lys, no membrane protein was radiolabeled when the labeling experiment was repeated using transport-defective L1210/R81 cells (Price & Freisheim, 1987). Figure 2 indicates that, unlike results with a cell homogenate supernatant from L1210/S cells, no labeling of an intracellular 38K protein can be detected in L1210/R81 cells. These data indicate that both the 46K–48K membrane carrier and the cytosolic 38K protein either are not expressed in MTX transport-defective L1210/R81 cells or are present as mutated species that do not bind the photoprobe and, presumably, MTX. When a rabbit antiserum raised against the purified folate receptor from human placental membranes was used to probe Western blots of plasma membrane proteins obtained from parent L1210 cells and L1210/R81 cells, a 48K band was obtained in both cases (Figure 5). This is presumably the MTX transport protein which appears to share antigenic determinants with the placental receptor. The 48K band is unlikely to represent a separate folate binding protein such as that seen in human KB cells since such a protein has not been found to be present in significant amounts in L1210 cells. In addition, the L1210/R81 cells exhibited an additional relatively minor band at 53K. The identity of this minor protein is unknown; it could be a higher molecular weight unprocessed precursor. Data from immunoblotting experiments (Figure 5) suggest that at least the 48K membrane protein is still present in the L1210/R81 cells albeit in a functionally inactive form. The possibility of a precursor–product relationship between the 48K and 38K proteins is currently being investigated.

On the basis of the data presented, a model for one-carbon-reduced folate/MTX transport is presented (Figure 6) as a working hypothesis. Following binding to the 46K–48K membrane carrier, the reduced folate or MTX molecule is translocated across the plasma membrane of the cell. Then, at the inner periphery of the membrane, the compound either is immediately bound by DHFR or, especially in the case of reduced folates whose intracellular target is not DHFR, is shuttled to other folate binding proteins via the 38K protein.

The finding that numerous one-carbon-reduced folates (such as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate) as well as MTX are all transported into L1210 cells via a common carrier was based upon influx inhibition studies. From these studies, we conclude that this carrier is the 46K–48K membrane-derived protein. The need for an intracellular reduced folate "shuttle" protein arises from the fact that, although the same carrier translocates different types of reduced folates (and MTX) across the membrane, the ultimate cytosolic target proteins for these compounds are different enzymes. The 38K protein, therefore, may mediate the distribution of folates, once inside the cell, to enzymes other than DHFR.

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Auracyanin, a Blue Copper Protein from the Green Photosynthetic Bacterium *Chloroflexus aurantiacus*†

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ABSTRACT: A small, type 1 blue copper protein has been isolated from the green photosynthetic bacterium *Chloroflexus aurantiacus*. This protein, named auracyanin, appears to be peripherally associated with the cytoplasmic membrane, with a midpoint potential of +240 mV and a molar extinction coefficient of ϵ_{596} = 2900 M^{-1} cm^{-1}. Auracyanin is isolated as a disulfide-bridged dimer with a monomer molecular mass of 12 800 Da. The isoelectric point of auracyanin is 4.0. ESR spectra exhibit rhombic distortion and give no indication of interaction between the coppers. The function of auracyanin is not yet known, although its redox properties are compatible with a role in photosynthetic and/or respiratory electron flow.

Copper-containing proteins have been isolated from a variety of sources both eukaryotic and bacterial. While the function of some of these copper proteins is unknown, many are thought to take part in some sort of electron-transfer process (Ryden, 1984). Copper proteins can have single or multiple copper centers with the copper in either the +1 or +2 state. Because Cu^{2+} has a d^9 electronic configuration, it is paramagnetic and

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