Affinity Labeling of Folate Transport Proteins with the *N*-Hydroxysuccinimide Ester of the γ-Isomer of Fluorescein–Methotrexate†

Jianguo Fan, Laura E. Pope,‡ Karin S. Vitols, and F. M. Huennekens*

Division of Biochemistry, Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, California 92037

Received November 5, 1990; Revised Manuscript Received January 28, 1991

ABSTRACT: Fluorescein–methotrexate, a derivative in which the fluorophore is linked via a diaminopentane spacer to either the α- or γ-carboxyl group of the glutamate moiety in the drug [Gapski et al. (1975) *J. Med. Chem. 18*, 526–528], has been synthesized by an improved procedure and separated by DEAE-Trisacryl chromatography into the α- and γ-isomers (α-F–MTX and γ-F–MTX). Each isomer was characterized by mass spectrometry, elemental analysis, absorbance spectrum, TLC, and reversed-phase HPLC. Identity of the isomers was established by the following enzymatic criteria: (a) γ-F–MTX (but not the α-isomer) was hydrolyzed at the pteroate–glutamate bond by carboxypeptidase G_2 to yield 4-amino-4-deoxy-10-methylpteroate and γ-glutamyldiaminopentane–fluorescein; and (b) γ-F–MTX was a much better inhibitor of human dihydrofolate reductase than the α-isomer (K_i values of 0.079 and 4.6 nM). α- and γ-F–MTX were comparable as inhibitors (K_i values of 1.6 and 0.6 μM) of the transport system for reduced folates and MTX in L1210 cells, but the transporter in *Lactobacillus casei* was inhibited only by the γ-isomer (K_i = 4.3 μM). The γ-isomer, therefore, was selected for *covalent* labeling of proteins. When *L. casei* folate transport protein (18 kDa) was treated with γ-F–MTX that had been activated with *N*-hydroxysuccinimide (NHS), the protein was readily visualized as a fluorescent band on SDS–PAGE electrophoretograms. The probe was also able to detect the transporter in membranes. SDS–PAGE analysis of a Triton X-100 extract of *L. casei* membrane fragments that had been pretreated with activated γ-F–MTX revealed only two fluorescent-labeled bands, viz., the 18-kDa transporter and an unidentified 33-kDa protein. The 43-kDa transporter for reduced folate compounds and MTX in L1210 cells was also labeled by this procedure but, because of its relatively low level, visualization required immunopurification, SDS–PAGE, and transfer to nitrocellulose, followed by immunoblotting with rabbit anti-fluorescein antibody/biotinylated goat anti-rabbit IgG/streptavidin–peroxidase conjugate. NHS-activated γ-F–MTX also facilitated visualization, via fluorescence microscopy, of folate transporters on individual L1210 cells. The validity of this procedure was demonstrated by the marked reduction in fluorescence when labeling was conducted in the presence of excess MTX or when a mutant subline (R81) down-regulated for the transporter was used. *L. casei* spheroplasts treated with NHS-activated γ-F–MTX were also fluorescent, and specificity was shown by reduced labeling in the presence of MTX. In this instance, however, the 33-kDa protein rather than the transporter appeared to be the labeled component.

Methotrexate (MTX),[1] which is used extensively in cancer chemotherapy, enters cells via a folate transport system and, after being polyglutamylated, exerts its cytotoxic action primarily through inhibition of dihydrofolate reductase [reviewed by Huennekens et al. (1987)]. To facilitate study of the interaction of MTX with the transporter or target enzyme, a fluorescent derivative was synthesized by coupling the α- or γ-carboxyl of the glutamate moiety of the drug, through a diaminopentane spacer, to fluorescein isothiocyanate. The product, fluorescein–methotrexate[2] [F-MTX; structure shown in Gapski et al (1975)], was comparable to MTX with respect to binding constants for *noncovalent* interaction with representative mammalian (L1210) and bacterial (*Lactobacillus casei*) dihydrofolate reductases and folate transport systems. Subsequent studies in other laboratories utilized this probe to visualize dihydrofolate reductase on electrophoretograms (Whiteley & Russell, 1979; Henderson et al., 1980) to examine binding of the drug to the enzyme (Degan et al., 1989), to quantitate or separate via flow cytometry MTX-resistant cells with elevated or reduced levels of the enzyme (Kaufman et al., 1978; Urlaub et al., 1985; Gaudray et al., 1986), and to identify cells that were resistant to MTX due to defective transport of the drug (Assaraf & Schimke, 1987). An analogue of F–MTX, in which lysine replaced the glutamate moiety and fluorescein was linked directly (i.e., without a spacer) to the ε-amino group of the lysine, was prepared by Rosowsky and colleagues (1982) and used to detect MTX-

† Supported by Outstanding Investigator Grant CA-39836 from the National Cancer Institute, National Institutes of Health, and by Grant CH-31Z from the American Cancer Society. Preliminary accounts of this work have been presented elsewhere (Fan et al., 1990a,b).

* Author to whom correspondence should be addressed.

‡ Recipient of postdoctoral fellowships from American Cancer Society and National Cancer Institute, National Institutes of Health. Present address: Lidak Pharmaceuticals, La Jolla, CA.

[1] Abbreviations: MTX, methotrexate; F–MTX, fluorescein derivative of MTX; α-F–MTX and γ-F–MTX, α- and γ-isomers of F–MTX; FITC, fluorescein isothiocyanate (isomer I); F–DAP, fluorescein diaminopentane; γ-F–glutamate, fluorescein derivative of glutamate; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; NHS, *N*-hydroxysuccinimide; NHSS, *N*-hydroxysulfosuccinimide; CP G_2, carboxypeptidase G_2, DMSO, dimethyl sulfoxide; DMF, dimethylformamide; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; NP-40, Nonidet P-40.

[2] Chemical Abstracts Index for γ-F–MTX: N^2-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-*N*-[5-[[[[3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)phenyl]amino]thioxomethyl]amino]-pentyl]-L-glutamine.

0006-2960/91/0430-4573$02.50/0

resistant cells with elevated levels of dihydrofolate reductase.

The original procedure (Gapski et al., 1975) for preparation of F–MTX was hampered by a lengthy purification procedure, persistent impurities, and low yields (ca. 15%). In addition, the α- and γ-isomers, which would be expected to result from the nonspecific route employed, were not separated. Other investigators improved the procedure by utilizing chromatography on DEAE-cellulose (Whiteley et al., 1986) or DEAE-Trisacryl (Gaudray et al., 1986) as a purification step and by adding acetonitrile to buffers (Gaudray et al., 1986) to minimize nonspecific adsorption of the product to the anion exchange. In the present investigation, the yield of F–MTX has been increased by use of the NHS ester of MTX for coupling with F–DAP, and the α- and γ-isomers have been separated by DEAE-Trisacryl chromatography and identified by enzymatic criteria. The isomers were examined as inhibitors of the *L. casei* and L1210 folate transport systems.[3] In each instance, γ-F–MTX had the higher affinity for the MTX binding site, and it was selected, therefore, for affinity labeling of the transport proteins. Treatment of *L. casei* membrane preparations or L1210 cells with γ-F–MTX whose free α-carboxyl group had been activated by conversion to the *N*-hydroxysuccinimide ester introduced into the transporters (18 and 43 kDa in *L. casei* and L1210, respectively) a covalently linked, fluorescent label that was stable to detergent extraction and SDS–PAGE. This procedure also allowed the L1210 transporter and an unidentified 33-kDa component in *L. casei* spheroplasts to be visualized by fluorescence microscopy.

Experimental Procedures

Materials. The following were obtained from the indicated commerical sources: FITC (isomer I), *N-t*-BOC-L-glutamic acid α-*tert*-butyl ester, phenylmethanesulfonyl fluoride, EDC, and NHS from Sigma; NHSS from Polysciences; 1,5-diaminopentane and Triton X-100 from Aldrich; CHAPS and NP-40 from Pierce; biotinylated goat anti-rabbit IgG, peroxidase–streptavidin conjugate, and streptavidin–agarose from Life Technologies; DEAE-Trisacryl from IBF Biotechnics; PEI cellulose TLC plates and C_{18} reversed-phase silica gel TLC plates from J. T. Baker; Centricon-10 microconcentrators from Amicon; C_{18} reversed-phase silica gel cartridges from Waters Associates; nitrocellulose from Gelman Sciences; [^{3}H]MTX, 18 Ci/mmol from Moravek; and pepstatin A, aprotinin, and leupeptin from US Biochemical Corp. The following gifts are gratefully acknowledged: MTX from Drs. Suresh Kerwar and Michael Boyd; CP G_2 from Drs. Joseph Bertino and Roger Sherwood; human dihydrofolate reductase (11 units/mg), isolated from *Escherichia coli* JM107 cells containing the cloned and overexpressed enzyme (Prendergast et al., 1988), and an MTX-resistant subline (R81) of L1210 cells down-regulated for transport (McCormick et al., 1981) from Dr. James Freisheim; and rabbit anti-fluorescein polyclonal antibody from Dr. Walter Dandliker. Dihydrofolate (Blakley, 1960) and *L. casei* folate transport protein (Henderson et al., 1977) were prepared by the indicated procedures. Propagation of L1210 cells (Henderson et al., 1978) and of *L. casei* cells, grown on low (1.0 nM) and high (1.0 μM) folate concentrations, and preparation of membrane fragments and spheroplasts from these cells (Henderson et al., 1977; Pope et al., 1989) have been described previously.

Synthesis of F–DAP. This intermediate was synthesized by the procedure of Gapski et al. (1975) with the following modifications. After condensation of FITC (750 mg) and diaminopentane (2.5 mL) in DMSO (6 mL), the product was collected by precipitation with 6 vol of acetone and then chromatographed on a 2 × 30 cm column of DEAE-Trisacryl with use of 0.1 M NH_4HCO_3 containing 20% CH_3CN as the eluant (flow rate, 2 mL/min; 50-mL fractions). Impurities eluted first, followed by F–DAP. Fractions containing F–DAP (monitored by TLC) were pooled and lyophilized. Acid precipitation of the product, used in the original procedure, was avoided since it produced some degradation: yield 60%; TLC (system A) R_f 0.32, (system B) R_f 0.60; HPLC (system A) retention time (rt) 15.0 min. Absorbance ratio (pH 13) A_{494}:A_{375} 12.6.

Synthesis of F–MTX. MTX (150 mg, 0.33 mmol), dried over P_2O_5 in vacuo, was dissolved in 2 mL of dry DMF. EDC (95 mg, 0.50 mmol) was added, and the solution was stirred in the dark under anhydrous conditions for 15 h at 4 °C. NHS (57 mg, 0.50 mmol) was added, and stirring was continued for another 6 h at 4 °C. F–DAP (54 mg, 0.11 mmol) was dissolved in 0.5 mL of DMF, and the slightly turbid solution was added dropwise to the above reaction mixture; the resulting clear solution was stirred further for 4 h at 4 °C. TLC verified that all of the F–DAP had reacted. Water (15 mL) was added, and the precipitate was collected by centrifugation (5000*g*, 20 min), washed with H_2O, and dissolved in 0.1 M NH_4OH. The solution (filtered through 0.45-μm nitrocellulose if turbid) was applied to a 2 × 30 cm column of DEAE-Trisacryl that had been equilibrated with 0.08 M NH_4HCO_3/20% CH_3CN. The column was eluted with 1400 mL of the same solvent (flow rate 0.6 mL/min), and the eluate containing unreacted MTX and degradation products was discarded. Elution was continued with 1500 mL of 0.2 M NH_4HCO_3/20% CH_3CN. Fractions (10 mL) were collected and examined by TLC (system A). Two forms of F–MTX, identified subsequently as the α- and γ-isomers (see Results and Discussion), eluted in that order from the column. Fractions 5–15 containing the α-isomer and fractions 90–125 containing the γ-isomer were pooled and lyophilized. α-F–MTX: yield (based on F–DAP) 7.4 mg (7.3%); TLC (system A) R_f 0.20, (system B) R_f 0.65; HPLC (system A) rt 20.4 min (shoulder, 20.1 min); A_{494}:A_{375} 5.2; MW 927 (Mass Spectrometry Laboratory, Research Institute of Scripps Clinic). Anal. Calcd. for $C_{46}H_{45}N_{11}O_9S{\cdot}3H_2O$ (982.0): C, 56.26; H, 5.23; N, 15.69; S, 3.26. Found: C, 55.40; H, 4.90; N, 15.38; S, 2.87 (Robertson Laboratory, Madison, NJ). γ-F–MTX: yield 45.0 mg (44%); TLC (system A) R_f 0.13, (system B) R_f 0.73; HPLC (system A) rt 22.9 min; A_{494}:A_{375} 5.2; MW 927. Anal. Found: C, 56.25; H, 5.06; N, 15.06; S, 2.85. MTX: TLC (system A) R_f 0.46; HPLC (system A) rt 16.8 min.

Synthesis of γ-F–Glutamate. (A) Enzymatic. γ-F–MTX (5.0 mg) was dissolved in 1 mL of 0.1 M Tris-HCl buffer, pH 7.3, containing 2 mM $ZnSO_4$, and CP G_2 (5 units) was added. The solution was incubated at 37 °C for 12 h and then heated at 100 °C for 1 min. *(B) Chemical. N-t*-BOC-L-Glu α-*tert*-butyl ester (5.0 mg, 17 μmol) was dissolved in 0.2 mL of dry DMSO. EDC (3.3 mg, 17 μmol) and NHS (2.0 mg, 17 μmol) in DMSO were added, and the mixture was stirred at room temperature for 3 h. F–DAP (6.0 mg, 12 μmol) was dissolved in 0.25 mL of DMSO and added to the above reaction mixture. Stirring was continued for another 3 h. The reaction mixture was added to 8 vol H_2O, and the precipitate

[3] L1210 cells contain two transport systems for folate compounds: (1) the low-affinity "reduced folate" system (K_t values for the preferred substrates 5-methyl- and 5-formyltetrahydrofolate and MTX in the micromolar range), and (2) the high-affinity system (K_t for folate in the nanomolar range). In the present study, "folate transport protein (or transporter)" refers to the low-affinity system.

was collected by centrifugation (5000*g*, 20 min), washed twice with H_2O, and air-dried. The solid was dissolved in 0.2 mL of dioxane; 0.1 mL of trifluoracetic acid was added, and after being stirred at room temperature for 3 h, the solution was neutralized by the dropwise addition of 5.0 M NaOH.

NHS Ester of γ-F–MTX. γ-F–MTX (20.0 mg) was dissolved in H_2O by addition of a minimum amount of 0.1 N NaOH and absorbed on a C_{18} reversed-phase silica gel cartridge. After extensive washing with H_2O, followed by 2% AcOH, γ-F–MTX (acid form) was eluted with 50% CH_3CN and lyophilized. A small amount (ca. 0.5 mg) of this material, dried over P_2O_5 in vacuo, was dissolved in 0.5 mL of dry DMSO; concentration was determined by adding an aliquot to 0.1 N NaOH and measuring the absorbance at 494 nm (ϵ_{mM} = 66). The solution was treated with a 7-fold molar excess of EDC for 45 min at room temperature and then with a 2-fold excess of NHS for 3 h at room temperature. Although generally used immediately for covalent labeling of proteins (as described below), the NHS ester of γ-F–MTX in DMSO could be stored at −20 °C for 2 days without a loss in activity.

HPLC. The procedure of Kuefner et al. (1989) was used, except that absorbance was monitored at 285 nm. System A: The mobile phase was formed with 5 mM tetrabutylammonium phosphate/10 mM $NH_4H_2PO_4$, pH 6.2 (pump A), and the same buffer in 50% CH_3CN, pH 7.2 (pump B); flow rate, 1 mL/min; sample size, 20 μL; gradient, 10% B for 3 min and 40% B for 2 min; linear gradient, 40–88% B for 20 min and then 88% B for 5 min. System B: The mobile phase consisted of 0.1 M NH_4OAc, pH 5.0 (pump A), and 0.025 M NH_4OAc in 80% CH_3CN, pH 5.5 (pump B); flow rate, 1 mL/min; sample size, 20–250 μL; gradient, 10% B for 2 min and 30% B for 3 min; linear gradient, 30–50% B for 30 min.

TLC. System A: PEI cellulose plates; 0.1 M NH_4HCO_3, pH 8.0, in 20% CH_3CN. System B: C_{18} reversed-phase silica gel plates; 5 mM tetrabutylammonium phosphate/10 mM $NH_4H_2PO_4$, pH 7.3, in 50% CH_3CN. Fluorescent compounds were visualized under UV light (254 nm).

SDS–PAGE. The general procedure of Laemmli (1970) was followed, by use of a 15% acrylamide slab gel (3-mm thickness): temperature, 4 °C; current, 25 mA. The gel was then placed on a firm black plastic support, sprayed with 7% acetic acid, and photographed (overhead UV illumination) with a Polaroid MP-4 land camera (Type 667 film). A Kodak gelatin filter (No. 15) was used to reduce the background. The gel was then stained with Coomassie blue, destained, and rephotographed under normal illumination.

Absorbance and Fluorescence Spectra. Measurements of the former were conducted with a Hewlett-Packard UV/VIS spectrophotometer, Model 8450A. Concentrations of α- or γ-F–MTX were determined by use of ϵ_{mM} = 66.0 at 494 nm (pH 13) (Whiteley et al., 1986); MTX concentrations were determined by use of ϵ_{mM} = 22.2 at 302 nm (pH 13) (Seeger et al., 1949). Fluorescence spectra were measured with a Perkin-Elmer fluorescence spectrophotometer, Model 650-40 (kindly provided by Dr. J. Whiteley).

Enzyme Assays. Carboxypeptidase G_2: The assay mixture contained, in 1.0 mL, 100 μM α-F–MTX, γ-F–MTX, or MTX and 0.2 mM $ZnSO_4$ and 0.1 M Tris buffer, pH 7.3. CP G_2 (0.048 units for F–MTX isomers and 0.012 units for MTX) was added, and the reaction was monitored via decreased absorbance at 320 nm: temperature, 37 °C. *Dihydrofolate reductase*: The assay mixture contained, in 1.0 mL, 500 μM NADPH, 5 μM dihydrofolate, and the indicated concentrations of α-F–MTX, γ-F–MTX, or MTX. Human dihydrofolate reductase (0.5 milliunits) was added, and the initial rate was determined via decreased absorbance at 340 nm with a Gilford spectrophotometer, Model 252: temperature, 37 °C.

MTX Transport. Transport of [^{3}H]MTX in *L. casei* (Henderon & Huennekens, 1974) or L1210 cells (Henderson & Zevely, 1980) was measured by the referenced procedures; α- or γ-F–MTX was present at the indicated concentrations.

Covalent Labeling of Dihydrofolate Reductase. Human dihydrofolate reductase (40 μg) in 0.08 mL of 0.1 M imidazole buffer, pH 6.4/1 mM EDTA/10% glycerol/10 mM β-mercaptoethanol was treated for 5 min at room temperature with 0.4 μL of 1.0 mM NHS ester of γ-F–MTX (or with 1.6 μL of 1.0 mM γ-F–MTX) in DMSO. The protein was precipitated by addition of 5 vol of cold acetone, recovered by centrifugation, and dissolved in a minimum volume (ca. 30 μL) of 2% SDS in 60 mM Tris-HCl buffer, pH 7.0, for SDS–PAGE analysis.

Covalent Labeling of L. casei Folate Transport Protein. L. casei folate transport protein (20 μg) in 0.08 mL of 5.0 mM $MgCl_2$/50 mM Hepes buffer, pH 7.5, containing 2% Triton X-100, was treated for 5 min at room temperature with 0.4 μL of 1.0 mM NHS ester of γ-F–MTX in DMSO. Recovery of the labeled protein and processing for SDS–PAGE was accomplished as described for dihydrofolate reductase. Labeling of the protein in membrane preparations was conducted by an alternate procedure. The membrane fraction was suspended in the above buffer at a density of 6400 Klett units. An aliquot (2.0 mL) of the suspension was treated with 10 μL of 1.0 mM NHS ester of γ-F–MTX in DMSO. After standing for 5 min at room temperature, the mixture was diluted with 8.0 mL of buffer (4 °C) and centrifuged (25000*g* for 10 min). The pellet was resuspended in buffer and the labeling procedure was repeated. After extensive washing with buffer, the pellet was treated with 2.0 mL of buffer containing 2.0% Triton X-100. The mixture was allowed to stand for 4 h at 4 °C and was then centrifuged (25000*g* for 30 min), and the extracted proteins were recovered from the supernatant and processed as described above.

Covalent Labeling of L1210 Folate Transport Protein. L1210 cells (8×10^9) suspended in 30 mL of 20 mM Hepes buffer, pH 7.4, containing 140 mM NaCl, 10 mM KCl, 2.0 mM $MgCl_2$, and 2.0 mM $CaCl_2$, were treated for 5 min (at 4 °C to minimize cell lysis) with 150 μL of 1.0 mM NHS ester of γ-F–MTX in DMSO. Cells were recovered by centrifugation, washed three times with buffer, and suspended in 45 mL of 1.0 mM $NaHCO_3$, pH 8, containing 2.0 mM $CaCl_2$, 5.0 mM $MgCl_2$, 20 μg/mL pepstatin A, 50 μg/mL aprotinin, 10 μg/mL leupeptin, and 1.0 mM phenylmethanesulfonyl fluoride. Cells were disrupted by homogenization (glass tube, zero-clearance Teflon pestle), and the plasma membrane fraction was isolated by sucrose gradient centrifugation (Henderson & Zevely, 1984). The membrane fraction was then washed three times with 5.0 mM $MgCl_2$/50 mM Hepes, pH 7.5, and the pellet was resuspended in 2.0 mL of the same buffer containing 20 μg/mL pepstatin A, 50 μg/mL aprotinin, 10 μg/mL leupeptin, 1.0 mM phenylmethanesulfonyl fluoride, and 2.0% Triton X–100. After standing for 5 h at 4 °C, the supernatant was recovered by centrifugation. The above procedure was repeated. The two membrane extracts were combined and diluted (if necessary) to give a protein concentration of approximately 1.5 mg/mL. Three milliliters of this solution was centrifuged (5000*g*, 34° fixed-angle rotor) in a Centricon microconcentrator to reduce the volume to ca. 0.3 mL. CHAPS (1% in 5.0 mM $MgCl_2$/50 mM Hepes, pH 7.5) was added to restore the original volume (3 mL), and the solution was centrifuged as above. This process was repeated

three times. The retentate was diluted with 1% CHAPS in a total volume of 1.5 mL and recovered by inverting the unit and centrifuging at 500*g* for 2 min. Rabbit anti-fluorescein antibody (100 μL) was added, and the solution was incubated with shaking for 16 h at 4 °C. To reduce nonspecific labeling, streptavidin–agarose (20 μL) that had been washed three times with 0.1% NP-40 in PBS was added, and after being shaken for 2 h at 4 °C, the solution was centrifuged (1000*g*, 2 min). The supernatant was treated with 25 μL of biotinylated goat anti-rabbit IgG for 2 h and then with 50 μL of streptavidin–agarose (washed as described above) for another 2 h. The streptavidin–agarose, recovered by centrifugation (1000*g*, 4 min) and washed five times with 0.1% NP-40 in PBS and one time with H_2O, was used directly for SDS–PAGE analysis.

Fluorescence Microscopy of γ-F–MTX-Labeled Cells. L1210 cells (5×10^6) were labeled with NHS ester of γ-F–MTX as described above, except that the volume of buffer was reduced to 0.2 mL and 1.0 μL of 1.0 mM NHS ester of γ-F–MTX was used. Cells were washed three times with buffer and then suspended in 50 μL of 80% glycerol. Five microliters of *p*-phenylenediamine (10 mg/mL in PBS) was added (to minimize photobleaching), and 2.0 μL of the cell suspension was applied to a glass slide with a cover slip and examined with a Zeiss Axiovert 10 inverted fluorescence microscope (100-W high-pressure mercury lamp; exciter barrier filter set Blue BP 485/20, LP 520/560; objective lens, Zeiss Plan Neofluar 40/0.75, pH 2). Photographs were taken with a 35-mm Zeiss microscope camera (Model MC 100) with Kodak TMAX 400 film. *L. casei* cells and spheroplasts were treated similarly except that the cell number was increased ca. 50-fold, and labeled cells were observed with a Zeiss Neofluar 100/1.30 oil objective lens.

Results and Discussion

Identification of α- and γ-Isomers of F–MTX. Both the faster and slower eluting components from the DEAE-Trisacryl chromatography step (see Experimental Procedures) had identical molecular weights of 927 (as determined by mass spectrometry), elemental composition, absorbance maxima at 494 and 375 nm (pH 13), and an absorbance ratio $A_{494}:A_{375}$ of 5.2. These data verified that each component contained equimolar amounts of MTX and fluorescein. From consideration of the synthetic route employed (activation of the carboxyl groups of MTX with EDC/NHS and coupling with F–DAP), it was evident that MTX had been derivatized on either the α- or γ-carboxyl group. The following enzymatic criteria were used to identify the faster and slower components as α-F–MTX and γ-F–MTX, respectively.

Carboxypeptidase G_2 provided a convenient enzymatic system for distinguising between these isomers. Previous work in this laboratory (Kuefner et al., 1989) had shown that derivatization of the α-carboxyl of MTX by addition of amino acids prevents cleavage by CP G_2 (at the pteroate–glutamate bond). When tested with CP G_2 in the spectrophotometric assay, α-F–MTX was inert but the γ-isomer was hydrolyzed at an appreciable rate (Figure 1). Although the rate of hydrolysis of γ-F–MTX was slower than that of MTX, this was attributed to the presence of the bulky fluorescein group.

Hydrolysis of γ-F–MTX by CP G_2 was verified by a separate experiment. HPLC analysis (system A) of the reaction mixture in Figure 1 revealed the presence of unreacted γ-F–MTX (rt = 22.8 min), 4-amino-4-deoxy-10-methylpteroate (rt = 13.6 min), and a fluorescent compound (rt = 17.6 min). The latter was identified as γ-F–glutamate (the expected product from CP G_2 mediated hydrolysis of γ-F–MTX) by HPLC comparison with an authentic sample prepared by chemical synthesis (see Experimental Procedure). The incomplete hydrolysis of γ-F–MTX (ca. 50%) shown in Figure 1 provided further evidence that the compound was a mixture of D- and L-enantiomers and that only the latter [like L-MTX (Cramer et al., 1984)] would be a substrate for CP G_2.

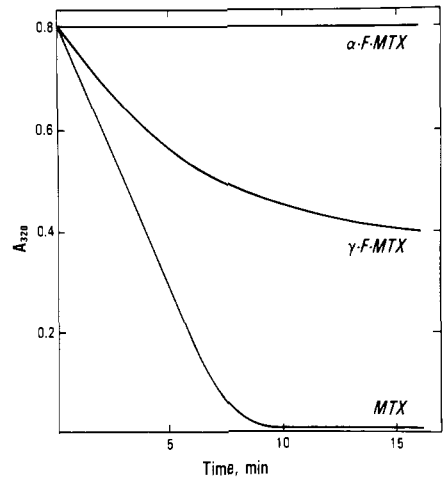

FIGURE 1: Hydrolysis of α- and γ-F–MTX by CP G_2. For details, see Experimental Procedures. Absorbance at 320 nm was monitored continuously as a function of time.

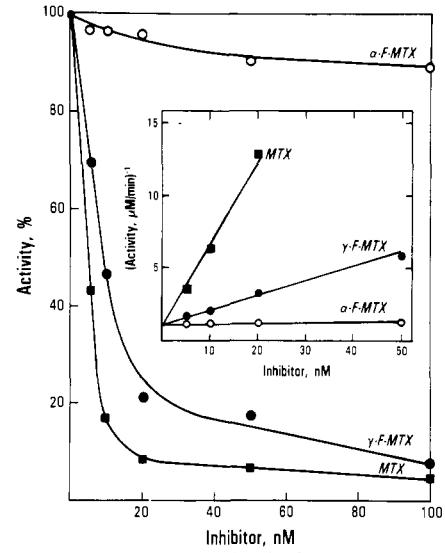

FIGURE 2: Inhibition of human dihydrofolate reductase by α- and γ-F–MTX. Spectrophotometric assay was used with indicated concentrations of α-F–MTX, γ-F–MTX, or MTX. Activity was calculated by use of $\Delta\epsilon_{mM} = 5.8$ at 340 nm (Osborn & Huennekens, 1958), and results are expressed as percent activity as a function of inhibitor concentration. The inset shows (activity, μM/min)$^{-1}$ vs inhibitor concentration. K_i values were calculated from slopes of curves by use of $K_m = 0.036$ μM for dihydrofolate (Prendergast et al., 1988).

Dihydrofolate reductase was also useful for identifying the components. X-ray crystallographic studies of the enzyme from several sources [reviewed by Freisheim and Matthews (1984)] have revealed that interaction of the α-carboxylate of MTX with an invariant arginine residue is a determinant of drug binding, and this had been confirmed by observations from several groups, e.g., Rosowsky et al. (1981), showing the α-derivatives of MTX are much less effective than the γ-counterparts as inhibitors of dihydrofolate reductase. When tested as inhibitors of human dihydrofolate reductase (Figure 2), γ-F–MTX (K_i = 0.079 nM) was almost as effective as MTX (K_i = 0.014 nM) while α-F–MTX was a much poorer inhibitor (K_i = 4.6 nM).

The overall yield of F–MTX and the relative amounts of the two isomers depended upon the reaction conditions em-

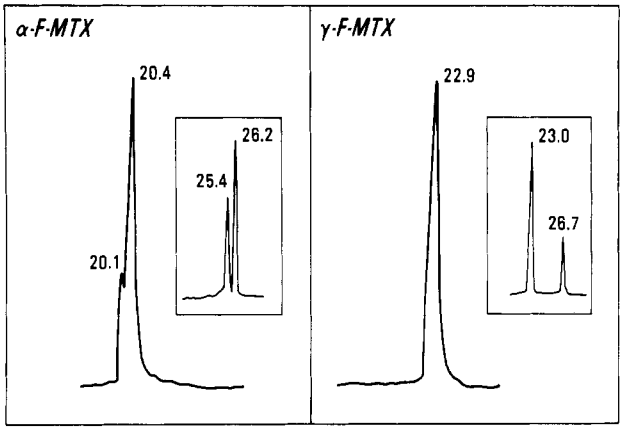

FIGURE 3: HPLC of α- and γ-F-MTX: (A) α-F-MTX, solvent system A; (B) γ-F-MTX, solvent system A; (insets) solvent system B. Retention times are indicated in minutes.

ployed. Under the standard conditions described in Experimental Procedures (i.e., 1.5-fold molar excess of EDC/NHS relative to MTX and reaction run in DMF at 4 °C), yields of the α- and γ-isomers were 7 and 44%, respectively. Approximately equal amounts of the isomers were obtained when a 2-fold molar excess of the dehydrating agents was used, although the total yield decreased to 39%. Both isomers were obtained as amorphous yellow powders; attempts to crystallize them have not been successful. They were quite stable, especially when the acid forms were stored dry at −20 °C and shielded from light. Each isomer was soluble (up to ca. 10 mM) in pH 7.0 buffers and generally insoluble in organic solvents except for DMF and DMSO. When examined by TLC, the isomers migrated as discrete fluorescent entities (see Experimental Procedures for R_f values in two solvent systems); no contaminants (F-DAP, MTX, or degradation products) were evident. In HPLC analysis, with system A (see Experimental Procedures), each isomer showed a single peak; retention times were 20.4 and 22.9 min for α- and γ-F-MTX (Figure 3). There was, however, a small shoulder (20.1 min) on the α-isomer peak, which suggested that two closely related compounds were present. Accordingly, other solvent systems were explored, and it was found (Figure 3, insets) that ammonium acetate/CH_3CN (system B) resolved α-F-MTX into a doublet (retention times 25.4 and 26.2 min) and, in addition, separated the γ-isomer into two peaks (retention times 23.0 and 26.7 min). This polymorphism was attributed to racemization of the L-glutamate moiety (producing L- and D-enantiomers of each isomer) that is known to occur when MTX is treated with coupling agents (Rosowsky & Yu, 1978; Kuefner et al., 1990). It cannot be due to multiple forms of the fluorescein moiety (e.g., ring-opened and ring-closed structures) or to the presence of some type II isomer as a contaminant in the FITC (type I) starting material because HPLC analysis (systems A or B) indicated only a single component in the F-DAP used for synthesis of α- and γ-F-MTX. However, since the enantiomers of α- and γ-F-MTX were not readily available on a preparative scale, subsequent experiments in this study were performed with the racemic mixtures of each isomer.

F-MTX as Covalent Label for Folate Transport Proteins. Previous studies have shown that F-MTX is a competitive inhibitor (K_i values of ca. 1 μM) of the transport of folate compounds in *L. casei* and L1210 cells (Gapski et al., 1975; Henderson et al., 1980). However, internalization of F-MTX is very slow (Henderson et al., 1980; Assaraf & Schimke, 1987), suggesting that the MTX moiety binds to the transport proteins but that the bulky fluorescein group impedes translocation of the compound through the membrane. In the present investigation, α-F-MTX and γ-F-MTX were evaluated for interaction with the L1210 and *L. casei* folate transport systems. As shown in Figure 4, the γ-isomer was slightly better than the α-isomer as an inhibitor of MTX transport in the eukaryotic cells. When the data were analyzed via a Dixon plot (Figure 4, inset), K_i values of 1.6 and 0.57 μM were obtained for α- and γ-F-MTX; these values are comparable with the K_t of 1.6 μM for MTX under the same conditions (Henderson, 1983). Somewhat different results were obtained, however, when the isomers were tested as inhibitors of the folate transport system in *L. casei* (Figure 5). γ-F-MTX, again, was a good inhibitor (K_i = 4.3 μM), but α-F-MTX was ineffective. The K_t value for MTX is 0.21 μM in this system (Henderson & Huennekens, 1974).

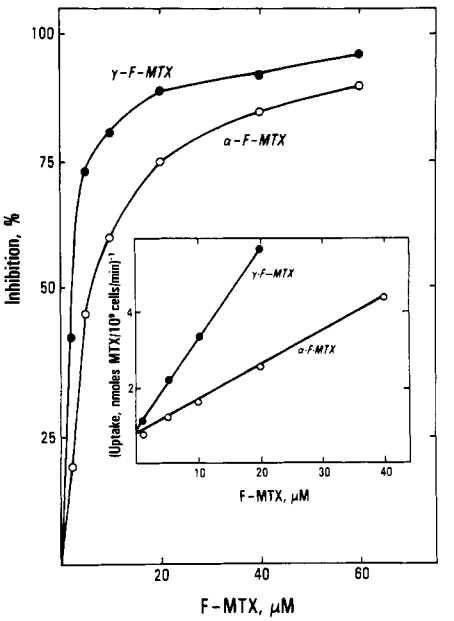

FIGURE 4: Inhibition of MTX transport in L1210 cells by α- and γ-F-MTX. Uptake of 5.0 μM [^{3}H]MTX was determined over the initial 10-min period in the presence of indicated concentrations of F-MTX isomers. Results (corrected for control uptake at 4 °C) are expressed as percent inhibition as a function of inhibitor concentration. The rate in the absence of inhibitors was 1.6 nmol per 10^9 cells per minute. The inset shows rate^{-1} vs inhibitor concentration. K_i values were calculated in Figure 2 by use of K_t = 1.6 μM for MTX (Henderson, 1983).

F-MTX has been employed previously as a *noncovalent* labeling agent for isolated or intracellular dihydrofolate reductases. Covalent attachment, however, would greatly increase the usefulness of this probe, since the labeled enzyme could be subjected to traumatic procedures such as SDS-PAGE, and it would also provide a means for labeling folate transport proteins or other enzymes that have a lower affinity than the dihydrofolate reductase for MTX. Since each isomer of F-MTX retains a free carboxyl, this group was suitable for activation, via conversion to the NHS ester, and subsequent linkage to protein. γ-F-MTX, which had proved to be a better substrate than the α-isomer for binding to dihydrofolate reductase and the transporters, was selected as the labeling agent. Human dihydrofolate reductase was utilized as a convenient model for development of this procedure. Treatment of the enzyme with 5 μM NHS ester of γ-F-MTX for 5 min at room temperature (see Experimental Procedures) yielded a fluorescent band (M_r = 22 kDa) that coincided with the Coomassie-stained band on SDS-PAGE (data not shown). No labeling was observed when the enzyme was treated with an even higher concentration (20 μM) of nonactivated γ-F-MTX

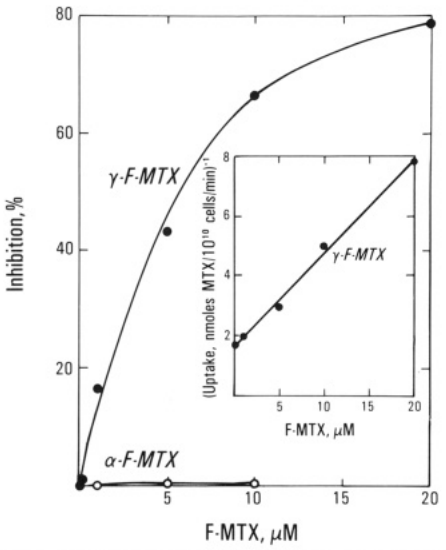

FIGURE 5: Inhibition of MTX transport in *L. casei* cells by α- and γ-F–MTX. Uptake of 1.0 μM [^{3}H]MTX was determined over the initial 5-min period in the presence of indicated concentrations of F–MTX isomers. Results (corrected for control uptake at 4 °C) are expressed as percent inhibition as a function of inhibitor concentration. The rate in the absence of inhibitors was 0.6 nmol per 10^{10} cells per minute. The inset shows rate^{-1} vs inhibitor concentration. K_i value for γ-F–MTX was calculated as in Figure 2 by use of K_t = 0.21 μM for MTX (Henderson & Huennekens, 1974).

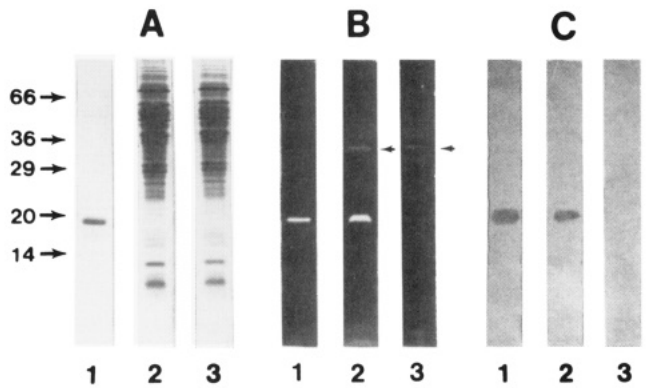

FIGURE 6: Covalent labeling of *L. casei* folate transport protein by the NHS ester of γ-F–MTX. The lanes show (1) *L. casei* transporter treated with 5.0 μM NHS ester of γ-F–MTX and subjected to SDS–PAGE; and (2 and 3) membrane preparations from cells grown on low (1.0 nM) and high (1.0 μM) concentrations of folate, respectively, and treated as above. Gels containing lanes 1–3 were photographed under UV light (panel B) and then stained with Coomassie Blue and rephotographed (panel A). Proteins on duplicate gels of lanes 1–3 were transferred to nitrocellulose and immunoblotted with rabbit anti-transport protein antibody/biotinylated goat anti-rabbit IgG/peroxidase–streptavidin conjugate (panel C).

or with 20 μM fluorescein whose carboxyl group had been exposed to activating conditions, i.e., preincubation with EDC/NHS in DMSO. The latter experiment verified that attachment of γ-F–MTX to the protein occurred via the α-carboxyl group of MTX rather than the carboxyl of the fluorescein.

Covalent Labeling of L. casei Folate Transport Protein. When the purified protein, solubilized in Triton X-100, was treated with NHS ester of γ-F–MTX and subjected to SDS–PAGE, a single fluorescent band (M_r = 18 kDa) was observed that coincided with the Coomassie band (Figure 6, lane 1 in panels A and B). The probe was also able to selectively label the transporter while the latter was present in membrane preparations (lane 2 in panels A and B). Also labeled in the membrane was a 33-kDa component (see arrow), which was present in variable amounts in different preparations of

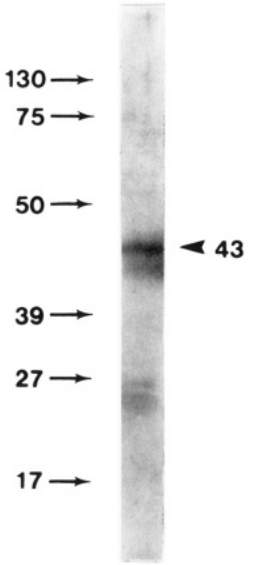

FIGURE 7: Covalent labeling of L1210 folate transport proteins by the NHS ester of γ-F–MTX. L1210 cells were labeled, the plasma membrane fraction was extracted with Triton, and the transporter in the extract was purified and concentrated as described in Experimental Procedures. The streptavidin–agarose gel containing the adsorbed transport protein was resuspended in SDS sample buffer and loaded directly on a 15% (3-mm) SDS–PAGE gel. After electrophoresis, the proteins were transferred to nitrocellulose and immunoblotted with rabbit anti-fluorescein antibody/biotinylated goat anti-rabbit IgG/peroxidase–streptavidin conjugate.

membrane fragments. The 18-kDa band was verified to be the folate transporter by Western blotting by use of an antibody directed against this protein (Pope et al., 1987). A positive response was obtained with the purified protein and with the membrane extract of cells grown on low folate (1.0 nM) (lanes 1 and 2 in panel C). With cells grown on high folate (1.0 μM), a condition known to repress synthesis of the folate transport protein (Henderson et al., 1976), no antibody-positive or fluorescent band at 18 kDa was observed (lane 3 in panels B and C) but the fluorescent 33-kDa component was still evident (lane 3 in panel B).

Covalent Labeling of L1210 Folate Transport Protein. When L1210 cells were treated with the NHS ester of γ-F–MTX under conditions used for labeling the *L. casei* transporter (except that the temperature was lowered to 4 °C), no fluorescent bands were visible on the electrophoretogram of the membrane extract (data not shown). To examine whether the probe was capable of binding to the transporter, a separate experiment was performed in which L1210 cells were treated with the NHS ester of γ-F–MTX (5 μM, 5 min, room temperature), washed thoroughly, and then tested for MTX transport activity. Inhibition (65%) was achieved under these conditions (data not shown). In contrast, no inhibition was observed when the cells were treated with nonactivated γ-F–MTX at the same concentration of with the EDC/NHS reagents in DMSO. It is likely, therefore, that failure to observe a labeled folate transport protein on the electrophoretogram was due to its relatively low level in L1210 cells (ca. 1% of the level of the *L. casei* transporter). To enhance the sensitivity of detection, the putative fluorescein-labeled protein in the membrane extract was purified with use of antibodies in combination with affinity adsorption on streptavidin–agrose (see Experimental Procedures) and then subjected to SDS–PAGE. After electrophoresis the proteins were transferred to nitrocellulose and immunoblotted with rabbit anti-fluorescein antibody, biotinylated goat anti-rabbit IgG, and peroxidase–streptavidin conjugate. As shown in Figure 7, this

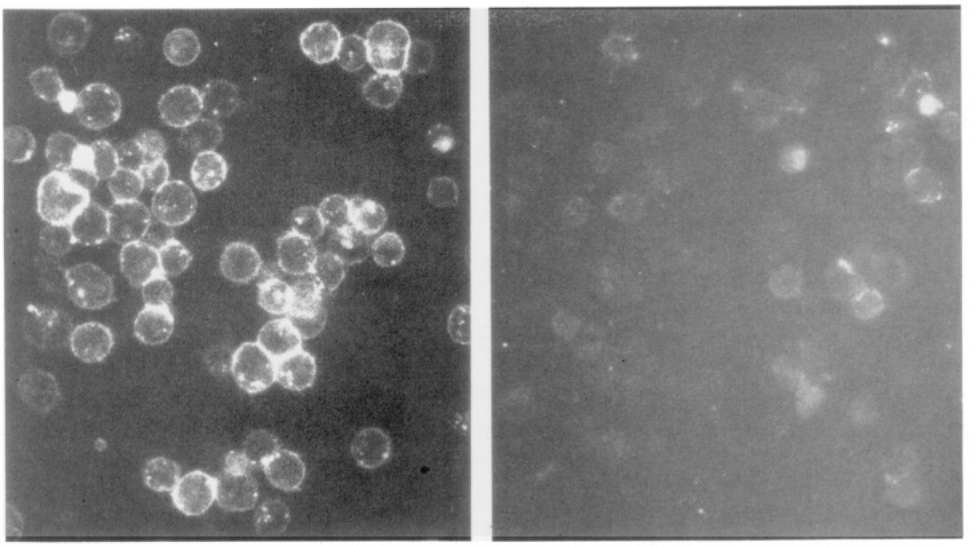

FIGURE 8: Fluorescence microscopy of L1210 cells labeled with the NHS ester of γ-F–MTX. The left panel shows L1210 cells labeled as described in Experimental Procedures. The right panel shows the control, transport-defective L1210 cells (R81 subline).

procedure visualized the the 43-kDa protein that previous investigators (Price & Freisheim, 1987; Yang et al., 1988) have identified as the folate transporter.

Fluorescence Microscopy of F–MTX-Labeled Membrane Proteins in Situ. Visualization of membrane proteins in individual cells is generally accomplished through the use of fluorescent-labeled antibodies. For this process to be effective, however, the protein must have suitable epitopes exposed above the membrane surface. In a previous study of the *L. casei* folate transporter (Pope et al., 1989), it was found that antibody prepared against the purified antigen was unable to detect (via immunoelectron microscopy) the transporter in situ unless the cell wall was removed and the membrane was permeabilized by brief treatment with Triton X-100. This observation, plus the extreme hydrophobicity of the protein, indicated that the latter was largely embedded in the membrane with only the folate binding site being exposed. The ability of NHS-activated F–MTX to selectively label folate transporters, even when the latter are embedded in membranes, suggested that the procedure could also be used to visualize the proteins in situ via fluorescence microscopy.

L1210 Cells. When L1210 cells were treated with the NHS ester of γ-F–MTX, washed thoroughly, and examined via fluorescence microscopy (see Experimental Procedures), the membranes exhibited a bright, yellow-green fluorescence (Figure 8, left panel). The specificity of the labeling procedure was established by control experiments in which fluorescence was greatly reduced when a transport-defective subline (R81) of L1210 cells was used (right panel) or when labeling of wild-type cells was conducted in the presence of excess MTX or thiamine pyrophosphate [a potent inhibitor of the transport system (Henderson & Zevely, 1983)] or when the NHS ester of γ-F–MTX was replaced with nonactivated γ-F–MTX; data for these latter three experiments are not shown.

Labeling of the folate transporter in situ has several potential uses, including (a) detection (via cytofluorography) and separation (via fluorescence-activated cell sorting) of cells up-regulated or down-regulated for the transporter, (b) monitoring changes in the level of transporter during cell growth or cell cycle or following treatments that may alter the proportion of transporters facing outward or inward, and (c) (at higher resolution) localization of the transporter on specific domains of the membrane.

L. casei Cells. Intact *L. casei*, treated with the NHS ester of γ-F–MTX under conditions used for L1210 cells (5.0 μM, 5 min, 4 °C), were not fluorescent. SDS–PAGE analysis of membrane extracts of these cells revealed that the 33-kDa protein, but not the 18-kDa transporter, was labeled (Figure 9, lane 1). Use of a higher concentration of the probe for a longer period of time (12 μM, 80 min, 4 °C) resulted in labeling of both proteins (lane 2). Even the more heavily labeled cells, however, were not fluorescent. Since it was possible that cell walls prevented visualization of fluorescence of the labeled proteins in intact cells, the latter were replaced by spheroplasts, which are devoid of cell walls. Following treatment with the NHS ester of γ-F–MTX (5 μM, 5 min, 4 °C), spheroplasts were highly fluorescent; labeling appeared to be localized in discrete segments along the rodlike structures (Figure 10). Specificity of labeling was shown by marked reduction in fluorescence when the procedure was conducted in the presence of a 400-fold excess of MTX or when the NHS ester of γ-F–MTX was replaced by nonactivated γ-F–MTX. Consistent with the results obtained with intact cells (Figure 9), SDS–PAGE analysis of spheroplast membranes showed that only the 33-kDa protein was labeled; use of higher concentrations of the probe for longer time periods (12 μM, 80 min, 4 °C) resulted in labeling of both the 33- and 18-kDa proteins (data not shown).

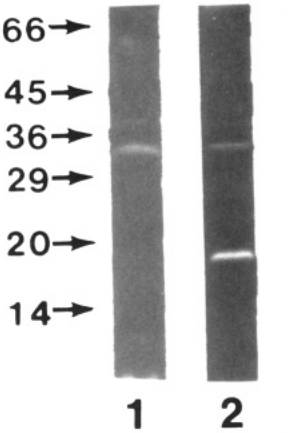

FIGURE 9: Labeling of the 18-kDa transporter and 33-kDa protein by treatment of *L. casei* cells with standard and more vigorous conditions. Detergent extracts were prepared from membrane preparations of the labeled cells and analyzed by SDS–PAGE; gels were photographed under UV light as described in Experimental Procedures (and Figure 6, panel B). In lane 1 conditions were 5 μM, 5 min, 4 °C. In lane 2 conditions were 12 μM, 80 min, and 4 °C.

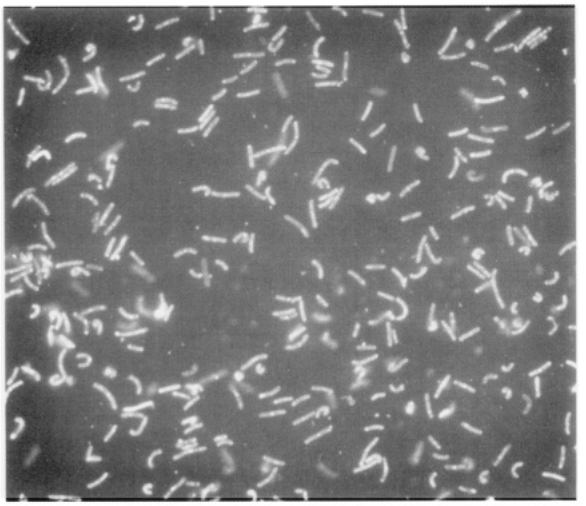

FIGURE 10: Fluorescence microscopy of *L. casei* spheroplasts labeled with the NHS ester of γ-F-MTX. Spheroplasts were prepared as described previously (Pope et al., 1989) and labeled with the NHS ester of γ-F-MTX under the conditions used for L1210 cells (see Experimental Procedures).

The results of this investigation indicate that the 33-kDa protein (a) has a reasonably high affinity for MTX, (b) is not repressed in cells grown on high folate, (c) is present in intact cells and spheroplasts and, to a variable extent, in membrane preparations, and (d) resides in a compartment between the cell wall and membrane. Further experiments to localize the 33-kDa protein and to determine its possible role in the transport process are currently in progress.

ACKNOWLEDGMENTS

We are indebted to Susan Burke for expert assistance in the preparation of the manuscript.

Registry No. DAP, 87328-05-0; FITC, 27072-45-3; α-F-MTX, 132884-72-1; γ-F-MTX, 104953-13-1; MTX, 59-05-2; γ-F-glutamate, 132884-73-2; γ-F-MTX NHS ester, 132884-74-3; diaminopentane, 462-94-2; dihydrofolate reductase, 9002-03-3.

REFERENCES

Assaraf, Y. G., & Schimke, R. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7154–7158.

Blakley, R. L. (1960) *Nature 188*, 231–232.

Cramer, S. M., Schornagel, J. H., Kalghatgi, K. K., Bertino, J. R., & Horvath, C. (1984) *Cancer Res. 44*, 1843–1846.

Degan, P., Carpano, P., Cercignani, G., & Montagnoli, G. (1989) *Int. J. Biochem. 21*, 291–295.

Fan, J., Pope, L. E., Vitols, K. S., & Huennekens, F. M. (1990a) *Adv. Enzyme Regul. 30*, 3–12.

Fan, J., Pope, L. E., Vitols, K. S., & Huennekens, F. M. (1990b) in *Chemistry and Biology of Pteridines 1989* (Curtius, H.-Ch., et al., Eds.) pp 1162–1165, Walter de Gruyter, Berlin.

Freisheim, J. H., & Matthews, D. A. (1984) in *Folate Antagonists as Therapeutic Agents* (Sirotnak, F. M., et al., Eds.) Vol. 1, pp 69–131, Academic Press, San Diego.

Gapski, G. R., Whiteley, J. M., Rader, J. I., Cramer, P. L., Henderson, G. B., Neef, V., & Huennekens, F. M. (1975) *J. Med. Chem. 18*, 526–528.

Gaudray, P., Trotter, J., & Wahl, G. M. (1986) *J. Biol. Chem. 261*, 6285–6292.

Henderson, G. B. (1983) in *13th International Cancer Congress, Part B, Biology of Cancer (1)*, pp 415–423, Alan R. Liss, Inc., New York.

Henderson, G. B., & Huennekens, F. M. (1974) *Arch. Biochem. Biophys. 164*, 722–728.

Henderson, G. B., & Zevely, E. M. (1980) *Arch. Biochem. Biophys. 200*, 149–155.

Henderson, G. B., & Zevely, E. M. (1983) *Arch. Biochem. Biophys. 221*, 438–446.

Henderson, G. B., & Zevely, E. M. (1984) *J. Biol. Chem. 259*, 4558–4562.

Henderson, G. B., Zevely, E. M., & Huennekens, F. M. (1976) *Biochem. Biophys. Res. Commun. 68*, 712–717.

Henderson, G. B., Zevely, E. M., & Huennekens, F. M. (1977) *J. Biol. Chem. 252*, 3760–3765.

Henderson, G. B., Zevely, E. M., & Huennekens, F. M. (1978) *Cancer Res. 38*, 859–861.

Henderson, G. B., Russell, A., & Whiteley, J. M. (1980) *Arch. Biochem. Biophys. 202*, 29–34.

Huennekens, F. M., Duffy, T. H., & Vitols, K. S. (1987) *NCI Monographs No. 5*, pp 1–8.

Kaufman, R. J., Bertino, J. R., & Schimke, R. T. (1978) *J. Biol. Chem. 253*, 5852–5860.

Kuefner, U., Lohrmann, U., Montejano, Y. D., Vitols, K. S., & Huennekens, F. M. (1989) *Biochemistry 28*, 2288–2297.

Kuefner, U., Esswein, A., Lohrmann, U., Montejano, Y., Vitols, K. S., & Huennekens, F. M. (1990) *Biochemistry 29*, 10540–10545.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

McCormick, J. I., Susten, S. S., & Freisheim, J. H. (1981) *Arch. Biochem. Biophys. 212*, 311–318.

Osborn, M. J., & Huennekens, F. M. (1958) *J. Biol. Chem. 233*, 969–974.

Pope, L. E., Minskoff, S. A., Chang, C.-M., Vitols, K. S., & Huennekens, F. M. (1987) *Anal. Biochem. 161*, 533–542.

Pope, L. E., Fan, J., Chang, C.-M., Minskoff, S. A., Vitols, K. S., & Huennekens, F. M. (1989) *Adv. Enzyme Regul. 28*, 3–11.

Prendergast, N. J., Delcamp, T. J., Smith, P. L., & Freisheim, J. H. (1988) *Biochemistry 27*, 3664–3671.

Price, E. M., & Freisheim, J. H. (1987) *Biochemistry 26*, 4757–4763.

Rosowsky, A., & Yu, C.-S. (1978) *J. Med. Chem. 21*, 170–175.

Rosowsky, A., Forsch, R., Uren, J., & Wick, M. (1981) *J. Med. Chem. 24*, 1450–1455.

Rosowsky, A., Wright, J. E., Shapiro, H., Beardsley, P., & Lazarus, H. (1982) *J. Biol. Chem. 257*, 14162–14167.

Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., & Hultquist, M. E. (1949) *J. Am. Chem. Soc. 71*, 1753–1758.

Urlaub, G., McDowell, J., & Chasin, L. A. (1985) *Somatic Cell Mol. Genet. 11*, 71–77.

Whiteley, J. M., & Russell, A. (1979) in *Chemistry and Biology of Pteridines* (Kislink, R. L., & Brown, G. M., Eds.) Vol. 4, pp 695–698, Elsevier North Holland, Inc., New York.

Whiteley, J. M., Webber, S., & Kerwar, S. (1986) *Prep. Biochem. 16*, 143–154.

Yang, C.-H., Sirotnak, F. M., & Mines, L. S. (1988) *J. Biol. Chem. 263*, 9703–9709.