Biotin Derivatives of Methotrexate and Folate

SYNTHESIS AND UTILIZATION FOR AFFINITY PURIFICATION OF TWO MEMBRANE-ASSOCIATED FOLATE TRANSPORTERS FROM L1210 CELLS*

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Biotin derivatives of methotrexate and folate (2-(biotinamido)ethyl-1,3'-dithiopropionylaminopentyl methotrexate and/or folate), in which carboxyl groups of the functional components are joined by a disulfide-containing spacer, have been synthesized, purified by DEAE-Trisacryl chromatography, and characterized by high pressure liquid chromatography and mass spectrometry. These bifunctional, dissociable probes were utilized for the single-step purification to homogeneity of two folate transport proteins (43 and 39 kDa) from L1210 cells. Treatment of the 39-kDa protein with peptide N-glycosidase F produced a smaller component (32 kDa); the 43-kDa protein, conversely, was unchanged by this procedure. When the 39-kDa transporter in intact cells was labeled with a fluorescein derivative of folate and then treated with phosphoinositol-specific phospholipase C, complete loss of fluorescence was observed. Alternatively, there was no change in fluorescence when the 43-kDa transporter was labeled with a fluorescein derivative of methotrexate and treated with the enzyme. These results indicate that the 43-kDa transporter is a nonglycosylated, integral membrane protein, whereas the 39-kDa counterpart is heavily glycosylated and anchored exofacially to the membrane by a glycosylphosphatidylinositol component.

Two separate transport systems for folate compounds and methotrexate (MTX) have been described in L1210 mouse leukemia cells. Parental cells propagated on micromolar concentrations of folate express a system in which the preferred substrates 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and MTX have K values in the range of 1-5 μM; folate, conversely, is a much poorer substrate (K > 100 μM) (1). Sublines derived from parental cells adapted to grow on nanomolar concentrations of folate or 5-formyltetrahydrofolate (2,3) are characterized by a much lower K for folate (<1 nm) (2); K values for 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and MTX (estimated from the reported Kp measurements (2,3)) also appear to be in the nanomolar range.

Although these transport systems have been characterized with respect to substrate specificity and kinetic characteristics, relatively little is known about the membrane-associated, binding proteins that play key roles in the translocation process. Labeling experiments have indicated that the transporter in parental cells (referred to hereafter as the “μM transporter”) has a molecular weight of 45-48 kDa (4,5). A slightly lower weight (42-44 kDa) has been reported for the “nm transporter” in the sublines described above (3). Low levels of these proteins (0.1-0.2 pmol/10⁶ cells for the μM transporter (1,6) and variable amounts up to ~7 pmol/10⁶ cells for the nm counterpart (3)) have hampered their purification. The present investigation was undertaken, therefore, to develop a rapid and efficient procedure for isolating these transporters. Biotin derivatives of MTX and folate have been synthesized and used to affinity label the proteins in intact L1210 cells. Streptavidin-agarose beads were employed to collect the labeled proteins from detergent extracts of the membranes, and the proteins were released by reduction of the S-S bond in the biotin-SS-MTX or -folate. The μM transporter (43 kDa) and nm transporter (39 kDa), isolated in pure form by this single-step procedure, have been characterized with respect to composition and mechanism for membrane attachment. This represents the first instance in which these transporters that appear to fulfill separate functions have been obtained from the same eukaryotic cell.

EXPERIMENTAL PROCEDURES

Materials—The following were obtained from commercial sources: NHSS-SS-biotin (Pierce Chemical Co.), streptavidin-agarose beads (Life Technologies), PVDF membranes (Millipore Corp.), Aurodye forte, and [14C]folate (30 Ci/mmol) (Amersham Corp.); the latter was purified prior to use by reversed-phase HPLC. Sources of other chemicals have been given previously (7). The following gifts are gratefully acknowledged: PI-PLC from Bacillus thuringiensis (strain 11807) (8) (Dr. S. Udendrief, Roche Institute of Molecular Biology) or from Bacillus subtilis transfected with the gene from B. thuringiensis (9) (Dr. M. Low, Columbia University), and peptide N-glycosidase F from Flavobacterium meningosepticum (10) (Dr. A. Tarentino, New York State Department of Health). 7-F-MTX was synthesized as described previously (11). Subline JF, up-regulated for the μM transporter, was selected by the following procedure. Parental cells propagated on micromolar concentrations of folate were used as the initial donor for subline JF; new cells were cloned by plating in 0.25% agarose. A colony exhibiting high fluorescence after treatment with 7-F-folate (7-isomers of fluorescein-MTX and 7-folate, respectively; 7-F-MTX and/or folate), in which carboxyl groups of the functional components are joined by a disulfide-containing spacer, have been synthesized, purified by DEAE-Trisacryl chromatography, and characterized by high pressure liquid chromatography and mass spectrometry. These bifunctional, dissociable probes were utilized for the single-step purification to homogeneity of two folate transport proteins (43 and 39 kDa) from L1210 cells. Treatment of the 39-kDa protein with peptide N-glycosidase F produced a smaller component (32 kDa); the 43-kDa protein, conversely, was unchanged by this procedure. When the 39-kDa transporter in intact cells was labeled with a fluorescein derivative of folate and then treated with phosphoinositol-specific phospholipase C, complete loss of fluorescence was observed. Alternatively, there was no change in fluorescence when the 43-kDa transporter was labeled with a fluorescein derivative of methotrexate and treated with the enzyme. These results indicate that the 43-kDa transporter is a nonglycosylated, integral membrane protein, whereas the 39-kDa counterpart is heavily glycosylated and anchored exofacially to the membrane by a glycosylphosphatidylinositol component.

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2 The abbreviations used are: MTX, methotrexate; biotin-SS-MTX and biotin-SS-folate, 2-(biotinamido)ethyl-1,3'-dithiopropionylaminopentyl derivatives of MTX and folate, respectively; r-F-MTX and r-F-folate, r-isomers of fluorescein-MTX and r-folate, respectively; NHSS, N-hydroxysuccinimide; NHSS-SS-biotin, sulfo- succinimidyl-2-(biotinamido)ethyl-1,3'-dithiopropionate; DAP, 1,5-diaminopentanote; MTX-DAP and folate-DAP, DAP derivatives of MTX or folate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; EDC, 1-ethyl-3-[3-dimethylaminoproyl]carbodiimide; NHS, N-hydroxysuccinimide; PI-PLC, phosphatidyl-inositol-specific phospholipase C; PVDF, polyvinylidene difluoride membrane; HPLC, high pressure liquid chromatography; MeSO₄, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazinethane-sulfonic acid; SDS, sodium dodeyl sulfate; PAGE, polyacrylamide gel electrophoresis; HBS, Hepes-buffered saline.

3 Subline JF, up-regulated for the μM transporter, was selected by the following procedure. Parental cells propagated on micromolar concentrations of folate were used as the initial donor for subline JF; new cells were cloned by plating in 0.25% agarose. A colony exhibiting high fluorescence after treatment with 7-F-folate was selected for propagation in folate-free RPMI medium supplemented with 2 nm 5-formyltetrahydrofolate and 5% fetal bovine serum that had been dialyzed and treated with activated charcoal. After 9 months, 5-formyltetrahydrofolate was decreased to 1 nm and, after an additional 8 months, cells were cloned by plating in 0.25% agarose. A colony exhibiting high fluorescence after treatment with 7-F-folate was selected for propagation in folate-free RPMI medium supplemented with 1 nm 5-formyltetrahydrofolate and 5% dialyzed human serum. The present investigation was undertaken, therefore, to develop a rapid and efficient procedure for isolating these transporters. Biotin derivatives of MTX and folate have been synthesized and used to affinity label the proteins in intact L1210 cells. Streptavidin-agarose beads were employed to collect the labeled proteins from detergent extracts of the membranes, and the proteins were released by reduction of the S-S bond in the biotin-SS-MTX or -folate. The μM transporter (43 kDa) and nm transporter (39 kDa), isolated in pure form by this single-step procedure, have been characterized with respect to composition and mechanism for membrane attachment. This represents the first instance in which these transporters that appear to fulfill separate functions have been obtained from the same eukaryotic cell.
and charcoal-treated fetal bovine serum.

**MTX-DAP—MTX (300 mg, 0.66 mmol),** dried over P₂O₅ in 3 ml of anhydrous Me₃SO was treated successively with EDC (151.8 mg, 0.79 mmol, room temperature, 1 h) and NHS (91.1 mg, 0.79 mmol, room temperature, 4 h). The solution was added dropwise with stirring to pre pared and lyophilized. Yield, 92.3 mg (83%). TLC: 0.48 HPLC: 10.1 min; fast atom bombardment mass spectroscopy, m/z = 539 (M+H)(™) (Mass Spectrometry Laboratory, Research Institute of Scripps Clinic). The following analysis was done at the Robertson Laboratory.

Ca₃H₉O₉N₂·3H₂O
Calculated: C 50.67 H 6.82 N 23.64
Found: C 50.90 H 6.55 N 23.15

| Biotin-SS-MTX—MTX (27 mg, 0.050 mmol), dried over P₂O₅ | and solubilized in 0.2 ml of anhydrous Me₃SO, NHS-S-S-biotin (30.4 mg, 0.050 mmol) in 0.4 ml of Me₃SO was added. | After 6 h at room temperature, the product was precipitated with acetone (10 ml), air-dried, dissolved in 1 ml of H₂O, followed by 0.05 M NH₄HCO₃ (10 ml fractions). Fractions 34—52 containing folate-DAP were pooled and lyophilized. Yield, 92.3 mg (M+H); tₑₐₚ = 22.7 at 306 nm (pH 13); HPLC, 18.8 and 17.0 min (before and after treatment with 2% P-mercaptoethanol). | Folate-DAP—Folate acid (200 mg, 0.45 mmol) was reacted with DAP, essentially as described for preparation of MTX-DAP. The DEAE-Trisacryl column was eluted with 200 ml of H₂O (discarded), followed by 0.05 M NH₄HCO₃ (10 ml fractions). Fractions 34—52 containing folate-DAP were pooled and lyophilized. Yield, 80.6 mg (23%); tₑₐₚ = 52.8 at 282 nm (pH 13); HPLC, 18.8 and 17.0 min. | The solution was added dropwise with stirring to 50 ml of 0.1 M NH₄HCO₃ and H₂O (30 min), followed by 0.1 M NH₄HCO₃, 20% CH₃CN (8-ml fractions). Fractions 135—141 containing biotin-SS-folate were pooled and lyophilized. Yield, 33 mg (36%); m/z = 915 (M+H); tₑₐₚ = 23.8 at 282 nm (pH 13); HPLC, 17.5 min. |
| NHSS Esters of Biotin-SS-MTX and Biotin-SS-Folate—Parent compounds were converted to their acid forms by the procedure described previously for fluorescein methotrexate (7), except that tetrahydrofuran (rather than CH₃CN) was used to elute the acid form | of DAP (0.773 ml, 6.60 mmol) in 1 ml of Me₂SO. After 3 h at room temperature, the product was precipitated with acetone (30 ml), air-dried, dissolved in 2 ml of H₂O, and chromatographed on DEAE-Trisacryl (2 × 30 cm, prewashed with 0.05 M NH₄HCO₃ and H₂O). The column was eluted with H₂O (0.6 ml/min; 6 ml fractions). Fractions 11—38 containing MTX-DAP were pooled and lyophilized. Yield, 111 mg (60%); tₑₐₚ = 58.9 at 282 nm (pH 13); HPLC, 12.7 and 18.0 min (before and after treatment with 2% P-mercaptoethanol). | of 1 M NH₄OH (final pH -7), and chromatographed on DEAE-Trisacryl column was eluted with 1 liter of 0.05 M NH₄HCO₃ and H₂O). The column was eluted with H₂O (100 ml, 45 min), followed by 0.1 M NH₄HCO₃, 20% CH₃CN (8-ml fractions). Pellets were collected by centrifugation (25,000 g, 30 min). | of 0.79 mmol, room temperature, 1 h) | of 150 mM sucrose, 10 mM glucose, 1% bovine serum albumin, 2.0 mM MgCl₂, 2.0 mM CaCl₂, | of 200 ml of H₂O (discarded), followed by 0.1 M NH₄HCO₃, 20% CH₃CN (8-ml fractions). Fractions 135—141 containing biotin-SS-folate were pooled and lyophilized. Yield, 33 mg (36%); m/z = 915 (M+H); tₑₐₚ = 23.8 at 282 nm (pH 13); HPLC, 17.5 min. |
| | of 0.05 M NH₄HCO₃ and H₂O). The column was eluted with H₂O (100 ml, 45 min), followed by 0.1 M NH₄HCO₃, 20% CH₃CN (8-ml fractions). Pellets were collected by centrifugation (25,000 g, 30 min). | | | of 200 ml of H₂O (discarded), followed by 0.1 M NH₄HCO₃, 20% CH₃CN (8-ml fractions). Fractions 135—141 containing biotin-SS-folate were pooled and lyophilized. Yield, 33 mg (36%); m/z = 915 (M+H); tₑₐₚ = 23.8 at 282 nm (pH 13); HPLC, 17.5 min. |
| NHSS Esters of Biotin-SS-MTX and Biotin-SS-Folate—Parent compounds were converted to their acid forms by the procedure described previously for fluorescein methotrexate (7), except that tetrahydrofuran (rather than CH₃CN) was used to elute the acid form of biotin-SS-folate from silica gel, and the solvent was removed by a rotary evaporator. Products were precipitated with acetone and dried at room temperature in vacuo. The acid form of biotin-SS-MTX or -folate (0.5 mg), dried over P₂O₅, was dissolved in 0.4 ml of anhydrous Me₃SO and treated sequentially with a 10-fold molar excess of EDC (room temperature, 30 min) and a 3-fold molar excess of NHSS (room temperature, 3.5 h). The resulting NHSS esters (concentrations determined spectrophotometrically) were used immediately. | Labeling and Affinity Isolation of μM Transporter from L1210 Cells—Parental L1210 cells (1.5 × 10⁹/ml) were collected by centrifugation (1000 × g, 5 min) and washed with HBS (20 ml Heps, pH 7.4, 140 mM NaCl, 10 mM KCl, 2.0 mM MgCl₂, 2.0 mM CaCl₂), and twice with labeling buffer (160 mM Heps, pH 7.4, 2 mM MgCl₂). Cells (~10⁶) suspended in 30 ml of labeling buffer were treated with 0.4 μmol of NHSS ester of biotin-SS-MTX on 100 ml of cold (4 °C) labeling buffer and shaken gently (5 min, 4 °C). Cells were recovered by centrifugation, washed once with labeling buffer, three times with HBS, and suspended in 20 ml of lysine buffer (1.0 mM NaHCO₃, pH 8.2, 20 mM CaCl₂, 5.0 mM MgCl₂, containing 20 μg/ml peptatin A, 0.05 μg/ml leupeptin, 10 μg/ml leupeptin and 1.0 mM phenylmethylsulfonyl fluoride). After 15 min at 4 °C, cells were centrifuged (3000 × g, 10 min), suspended in 45 ml of lysine buffer, and homogenized 20 times in a glass tube with a zero-clearance Teflon pestle. The plasma membrane fraction was isolated by sucrose gradient centrifugation (12), washed three times with 50 ml Heps (pH 7.5), 50 mM MgCl₂, and 0.1% Nonidet P-40 (5 min), and resuspended in 8 ml of buffer containing protease inhibitors (see above) and 1% CHAPS. After standing at 4 °C for 6 h, the supernatant was recovered by centrifugation (25,000 × g, 30 min). Streptavidin-agaroose beads (125 μl, bed volume) (prewashed three times with 50 mM Heps, pH 7.5, 5 mM MgCl₂, 0.1% Nonidet P-40 and once with 50 mM Heps, pH 7.5, 5 mM MgCl₂, 1.2% CHAPS) were added, and the suspension was shaken gently (6 h, 4 °C). Beads were recovered by centrifugation (1000 × g, 5 min), washed three times with 50 ml Heps (pH 7.5), 5 mM MgCl₂, and resuspended in 8 ml of buffer containing protease inhibitors (see above) and 1% CHAPS. After standing at 4 °C for 6 h, the supernatant was recovered by centrifugation (25,000 × g, 30 min). | Synthesis and Characterization of Biotin-SS-MTX and Biotin-SS-Folate—Avidin-biotin technology has been utilized extensively for protein purification and, more recently, recovery of biotinylated proteins from immobilized avidin has been facilitated by the commercial availability of spacers (linking biotin to the protein) containing readily dissociable groups. In the present investigation, biotin probes were prepared via a two-stage sequence. MTX or folate was reacted with DAP, and the products were joined to a biotin derivative in which the valeric acid moiety had been extended by addition of an -HN(CH₃)₂S-(CH₂)₄-COOH group (see “Experimental Procedures”). The structure of biotin-SS-MTX is shown in Fig. 1. Products purified by chromatography on DEAE-Trisacryl were obtained in overall yields of 35—50%. Each displayed a single HPLC peak and had the appropriate molecular weight as determined by fast atom bombardment mass spectrometry.

**RESULTS AND DISCUSSION**

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Fig. 1. Biotin-SS-MTX (l-isomer).

Fig. 2. SDS-PAGE of μM and nM folate transporters. Labeling, purification, and SDS-PAGE analysis of the transporters are described under "Experimental Procedures." Lanes A and B, parental cell membrane extract (200 μg) before and after treatment with streptavidin-agarose beads; lanes C and D, μM transporter (5 μg) purified in the presence and absence of protease inhibitors; lane E, nM and μM transporters (20 μg) co-purified from the JF subline. Molecular weights of standard proteins and transporters are indicated by arrows (left and right, respectively).

Fig. 3. Treatment of folate transporters with peptide N-glycosidase F. Details are given under "Experimental Procedures." Lanes A and C, untreated μM and nM transporters; lanes B and D, μM and nM transporters after treatment with enzyme.

Fig. 4. Treatment of folate transporters in situ with PIP-PLC. JF subline cells were labeled noncovalently with τ-F-folate (Panel A) and then treated with PIP-PLC (Panel B). Parental cells were labeled covalently with τ-F-MTX (Panel C) and then treated with the enzyme (Panel D).

Mercaptoethanol-mediated cleavage of the disulfide bond in these compounds was verified by HPLC.

Cell Lines and Transport Systems—Parental L1210 cells used in this study displayed the characteristic kinetic constants of the μM transport system (1). Although present at a low level, the transporter is readily visualized via fluorescence microscopy after treatment of the cells with the NHS ester of 5′formyltetrahydrofolate (see "Experimental Procedures"), exhibited appreciable binding of [3H]folate (6.4 pmol/10⁶ cells) and were highly fluorescent after treatment with the fluorescein-folate probe. These cells also retained the μM transporter (see below).

Isolation of the μM and nM Transporters—Parental cells were treated with the NHSS ester of biotin-SS-MTX, which positioned itself in the substrate-binding site of the μM transporter and attached covalently. A detergent extract of the cell membranes was exposed to streptavidin beads and, although this produced no discernible change in the protein profile visualized by SDS-PAGE (cf. Fig. 2, lanes A and B), subsequent treatment of the beads with dithiothreitol released a single protein that appeared as a sharp band on the electrophoretogram (lane C). The absence of other proteins indicated the highly selective nature of this purification procedure. The overall yield of pure transporter is estimated to be 10–20 μg from 10¹⁰ cells. The molecular weight (~43 kDa) of the transporter agreed with the value from previous investigations in which parental cells were treated with the NHS ester of [3H]aminopterin (4) or an ¹²⁵I-labeled derivative of the lysine analog of MTX (5), and the transporter was identified by SDS-PAGE/radioautography in the mixture of proteins in membrane extracts. A similar value was also obtained by treating cells with the NHS ester of τ-F-MTX and visualizing the transporter with a double-antibody technique (7, 16).

Repetition of the above procedure in the absence of protease inhibitors during cell lysis and membrane isolation produced an additional band (36 kDa) (lane D). This entity, which was observed previously by Henderson and Zevely (12), appears to be a proteolytic fragment of the μM folate transporter (4). Microsequencing of the 43- and 36-kDa proteins by Edman
The 43-kDa entity. Data in Fig. 2 also suggest that the folate binding site resides in the 36-kDa domain.

The nM folate transporter was isolated from the JF subline, using the NHSS ester of biotin-SS-folate as the probe. SDS-PAGE examination of the streptavidin eluate revealed the transporter as a heavy, diffuse band (39 kDa) accompanied with binding data presented earlier. No change in the molecular weight of the nM transporter was observed when protease inhibitors were omitted during purification (data not shown). Thus, the JF subline contains both the nM and μM transporters, in contrast to parental cells which express only the μM transporter.

Characterization of the μM and nM Transporters—The presence of carbohydrate in the transporters was assessed by treatment of the proteins with specific enzymes. Peptide N-glycosidase F (N-glycanase), which hydrolyzes asparagine-sugar linkages, had no effect on the μM transporter, as shown by the unchanged molecular weight of the protein (Fig. 3, lanes A and B). The 32 kDa band in lane B is peptide N-glycosidase F (10). In contrast, treatment of the nM transporter with the enzyme caused complete disappearance of the 39 kDa band (lanes C and D). The absence of any new band in lane D and the intensification of the 33 kDa band (cf. lanes D and B) suggests that the deglycosylated nM transporter co-migrates with peptide N-glycosidase F. Similar susceptibility to carbohydrate-removing enzymes have been obtained with counterparts of the nM transporter from KB cells (17) and human placenta (18).

Use of PI-PLC in conjunction with fluorescein derivatives of folate and MTX provided evidence regarding membrane anchorage of the transporters. When the JF subline was labeled noncovalently with γ-F-folate, washed, and then treated with PI-PLC, the nM transporter was released from the cells as shown by complete loss of fluorescence (Fig. 4, Panels A and B). In control experiments, no diminution in fluorescence was observed when enzyme was omitted, and labeled cells treated with enzyme did not become fluorescent upon re-exposure to the probe (data not shown). In contrast, when parental cells (containing the μM transporter) were labeled with γ-F-MTX and treated with PI-PLC, the fluorescence intensity remained constant (Panels C and D). Other investigators have shown previously that PI-PLC released the μM transporter (labeled with [3H]folate) from MA-10 cells (19) and converted the membrane form of the transporter from KB cells to its soluble counterpart (20). Thus, the nM folate transporter from L1216 cells is also exofacial on the membrane and anchored by a glycosylphosphatidylinositol component. These properties, plus its molecular weight and loss of carbohydrate after treatment with peptide N-glycosidase F, provide strong evidence that it closely resembles the "high affinity" transporter from human placenta (18), CaCo-2 cells (19), and KB cells (20). The L1216 μM transporter, conversely, appears to be an integral membrane protein lacking a glycosylphosphatidylinositol anchor and devoid of carbohydrate. Its counterpart in K562 cells, however, has a much higher molecular weight (78 kDa) and contains carbohydrate (21).

The methodology described in this paper is applicable to the purification of other membrane-associated folate transport proteins. It may also provide a means for the rapid isolation of folate-dependent enzymes.

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REFERENCES