Metabolic Turnover of Methotrexate Polyglutamates in Lysosomes Derived from S180 Cells

DEFINITION OF A TWO-STEP PROCESS LIMITED BY MEDIATEDLYSOSOMALPERMEATIONOFPOLYGLUTAMATESANDACTIVATINGREDUCEDSULFHYDRYLCOMPOUNDS*

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José R. Barrueco, Donna F. O’Leary, and Francis M. Sirotnak†
From the Laboratory for Molecular Therapeutics, Program in Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center and Graduate School of Medical Sciences, Cornell University, New York, New York 10021

Transport and metabolic turnover of methotrexate (MTX) polyglutamates were examined in lysosomes derived from S180 cells. These studies extend prior work from this laboratory (Barrueco, J. R., and Sirotnak, F. M. (1991) J. Biol. Chem. 266, 11732–11737) which described basic properties of a facilitative transport system in lysosomes capable of mediating intralysosomal accumulation of MTX polyglutamates. In the present report, we show that the rate of turnover of MTX polyglutamates in lysosomes, which releases MTX in the extralysosomal space, is limited by the extent of mediated intralysosomal accumulation of the polyglutamate and reduced sulfhydrolys that activate the enzyme folypolyglutamate hydrolase. Evidence is presented that cysteine functions as the naturally occurring reduced sulfhydryl compound in lysosomes being equipotent to 2-mercaptoethanol as an activator of folypolyglutamate hydrolase. Folypolyglutamate hydrolase in permeabilized lysosomes from S180 cells exhibited a low pH optimum characteristic of a lysosomal enzyme, was activated at concentrations of reduced sulfhydryl at 0.1 mM and above, and exhibited \( K_m \) values in the range of 0.2–3 \( \mu \)M that decreased with increase in polyglutamate chain length. Values for \( K_m \) for MTX polyglutamates of folypolyglutamate hydrolase activity were 100–200-fold lower than values for \( K_i \) or \( K_f \) for facilitated intralysosomal transport, whereas capacities for both processes were similar. This relationship between the kinetic properties of each process ensures efficient hydrolysis of MTX polyglutamates within the lysosome.

Methotrexate (MTX)* is an analog of the folic acid vitamins useful in the treatment of neoplasia. Its structural similarity to the folates and potent inhibition of the enzyme, dihydrofolate reductase, evokes profound effects on biochemical reactions involving these vitamins, including de novo synthesis of thymidine and purines in tumor cells. Conversion of MTX to MTX polyglutamates (MTXPGs), a process (reviewed in Refs. 1–4) that involves the sequential \( \gamma \) linkage of glutamyl residues to the terminal glutamyl moiety of the molecule, occurs in the cytoplasm and is catalyzed by the enzyme folypolyglutamate (FPG) synthetase. This process has been well characterized (1–4), as it also converts the naturally occurring folates to the corresponding polyglutamates. The higher polyglutamates, which appear to be pharmacologically more effective than the parent compound, are also for the most part readily retained inside the cell (1–4). Metabolic breakdown of polyglutamates is catalyzed by the counterpart enzyme, FPG hydrolase. This enzyme is of lysosomal origin, exhibits an acidic pH optimum, and requires the presence of reduced sulfhydryls for full expression of activity (2, 5–7). In effect, these two events occur in separate compartments in the cell.

Recently, we described (8) basic properties of a facilitative transport system that is present at the level of the lysosomal membrane and is capable of mediating the transfer of MTXPGs from the cytosolic to the lysosomal compartment of the cell. This transport system is stimulated by the presence of potassium and magnesium ions in the medium and recognizes longer chain length polyglutamates of MTX with increasing affinity (8). The present report focuses on the process of MTXPG turnover in lysosomes purified from S180 cells and the relationship between transport and hydrolysis in these organelles. The data show that there are two separate and independent events in a process that is limited by the delivery of MTXPGs and reduced sulfhydryl compounds to the intralysosomal space. In addition, evidence is presented to support the view that cysteine may function as the naturally occurring reduced sulfhydryl that is capable of activating lysosomal FPG hydrolase.

EXPERIMENTAL PROCEDURES

Lysosomes were prepared from S180 cells as described previously (8, 9). In short, cells were gently homogenized in sucrose/EDTA buffer (250 mM sucrose, 1 mM EDTA, pH 7.6, with Tris base) and the homogenate submitted to a series of centrifugation steps to yield a crude granular fraction. This fraction was further purified in 28% Percoll containing 250 mM sucrose, 1 mM EDTA, and 20 mM MOPS/Tris, pH 7.2. The resulting final lysosomal fraction was identified by its content of \( \beta \)-hexosaminidase (8, 9), washed, and resuspended in a small volume of sucrose/MOPS buffer (250 mM sucrose, 20 mM MOPS/Tris, pH 7.2) and immediately used in experiments.

For transport experiments (8), aliquots of lysosomal suspension and KCl/MgCl\(_2\)/MOPS buffer (166.7 mM KCl, 8.3 mM MgCl\(_2\), 20 mM MOPS, pH 7.2) were added to the cell suspension to give final concentrations of 100 mM potassium, 5 mM MgCl\(_2\), and 20 mM MOPS. After 10 min, the reaction was stopped by the addition of 6% perchloric acid, and the samples were centrifuged to remove the cells. The supernatant fraction was then assayed for MTX and MTXPGs using high-performance liquid chromatography.

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† To whom correspondence and reprint requests should be addressed: Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

‡ The abbreviations used are: MTX, methotrexate; FPG, folypolyglutamate; MTXPG, methotrexate polyglutamate; MTX + \( G_n \), methotrexate polyglutamate containing \( n \) number of additional glutamyl residues; MRS 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MET, 2-mercaptoethanol; CYS, Cysteine; \( \beta \)-hex, \( \beta \)-hexosaminidase; HPLC, high performance liquid chromatography.
20 mm MOPS/Tris, pH 7.2) containing the [3H]MTX + G1 substrate (methotrexate with 1 additional glutamyl residue; specific activity 100-150 dpm/pmol) were preincubated at 37 °C and mixed at a 2:3 ratio (lysosomal suspension:KCl/MgCl2/MOPS buffer), to initiate intralysosomal accumulation. Final concentrations of salts and sucrose in the mixture were 100 mM sucrose, 100 mM KCl, 5 mM MgCl2, 20 mM MOPS/Tris, pH 7.2. At the indicated times aliquots were removed from the experimental mixture, filtered, and washed as described previously (8). Radioactivity accumulated in lysosomes was corrected for nonspecific surface binding, converted to mass units, and expressed as a function of lysosomal integrity (9).

In experiments measuring efflux of substrate, lysosomes were first loaded with 800 μM substrate at 37 °C for 10 min and subsequently diluted 16-fold with drug-free buffer containing 100 mM sucrose, 100 mM KCl, 5 mM MgCl2, and 20 mM MOPS/Tris, pH 7.2. The resulting mixture was sampled, filtered, and washed at specified times as described above. Efflux of substrate was determined from a comparison of the intralysosomal content of total radioactivity initially and after various periods of incubation at 37 °C.

In experiments where the effect of cysteine or MET was measured during uptake, lysosomes were preincubated at 37 °C for 5 min with the substrate at a concentration of 10 mM. When these experiments were performed in preloaded lysosomes, cysteine, MET, or immobilized cysteine were added after the substrate load, at the time of dilution. Measurement of substrate hydrolysis as evidenced by MTX production was made by HPLC analysis. In these experiments mixtures prepared as above were sampled at specific times, the aliquots diluted in 300-500 μl of ice-cold sucrose/MOPS buffer and then rapidly transferred to a boiling-water bath for 5 min to terminate the reaction. After cooling, samples were cleared by centrifugation and analyzed by HPLC. Separation of substrate and product was done by reverse-phase chromatography (10) employing a linear gradient of 5-22% acetonitrile in 0.1 M ammonium acetate, pH 5.1. Peak detection was done using the Maxima 820 software system available from Waters Division of Millipore.

In some experiments, FPG hydrolase activity was measured using lysosomal preparations permeabilized by the addition of 0.1% Triton X-100. These experiments aliquots of lysosomal suspensions were incubated for 20 min at 37 °C in medium containing 10 mM MET, 0.1% Triton X-100, and 0.1 M each of MES and acetate, titrated to pH 5.5 with NaOH. Substrate was added at the appropriate concentrations depending on experimental design. The reaction was terminated by rapid boiling and samples analyzed by HPLC. Determination of the pH profile for FPG hydrolase activity was made in a buffer mixture containing 0.1 M concentrations of formate, acetate, MES, and HEPES. pH was adjusted to the appropriate levels by titration with either HCl or NaOH. Values for K, and Vmax, for hydrolysis were generated by linear regression analysis performed by the method of least squares. Data were expressed as a function of velocity versus concentration and derived from the results of three to five separate experiments averaged.

β-Hexosaminidase activity was measured at 37 °C and pH 4.6 for 5 min as described previously (8, 11, 12) using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. One unit of β-hexosaminidase activity was defined as the amount of enzymatic activity necessary to generate 1 nmol of product/min at 37 °C in the above assay. In transport experiments where time points exceeded 5 min of incubation, a preincubation step in the appropriate buffer preceded the 5-min β-hexosaminidase assay so that the total incubation time for the lysosomal aliquot to be assayed equaled 5 min. The enzyme is activated by reduced sulfhydryls such as mercaptoethanol. We then measured MTX + G1 hydrolysis in intact lysosomes under the identical conditions used to measure uptake and found that MTX production increased linearly over time for the duration of the experiment. Furthermore, as shown in Fig. 1, the rate of MTX production did not exceed the rate of initial uptake of substrate measured in the absence of MET. In contrast, the rate of MTX production in the absence of MET, although linear with time, was about 10-12-fold lower than the rate observed in the presence of MET. Although effective in its ability to activate FPG hydrolase in vitro, MET is not a known natural component of the cell.
The work of others (14-17), demonstrating lysosomal transport of cysteine, as well as its ability to participate in sulfhydryl-requiring reactions in lysosomes, provides strong indication that the amino acid, cysteine, is the natural activator of FPG hydrolase in the cell. We compared MET and cysteine for their ability to activate FPG hydrolase in intact lysosomes. Data in Fig. 2A show the large difference in accumulation of MTX with time in the presence or absence of either 5 mM MET or cysteine. The companion data in Fig. 2B show that both MET and cysteine stimulated FPG hydrolase in a concentration-dependent manner with the hydrolytic response similarly maximized at 5 mM or above. Moreover, the same concentration responses were obtained (data not shown) when the lysosomes were permeabilized by 0.1% Triton X-100.

Using cysteine as the reduced sulfhydryl, the phenomenon observed (Figs. 1 and 2) when [3H]MTX + G1 uptake was measured in the presence of MET was further examined. In these experiments, we measured the retention and hydrolysis of [3H]MTX + G1 in lysosomes preloaded with this permeant and subsequently incubated in the presence and absence of 5 mM free cysteine or cysteine immobilized by attachment to cellulose. The latter is impermeable to the lysosomal membrane but fully potent with respect to reduced sulfhydryl equivalence and activation of solubilized FPG hydrolase (data not shown). In each case, lysosomes were loaded with substrate in the absence of sulfhydryls and subsequently diluted with 16-fold buffer containing the specified agent. The results are given in Fig. 3. Rapid efflux of [3H]MTX + G1 from lysosomes could be demonstrated in these experiments. This was shown by the loss from lysosomes over time at 37 °C of [3H]MTX + G1 in the absence of reduced sulfhydryls. No efflux of [3H]MTX + G1 occurred at 4 °C during the same period of incubation, and no difference in efflux was found when higher dilutions of lysosome preparation were used to initiate efflux. However, consistent with data presented above (Fig. 1), [3H]MTX + G1 was lost more rapidly from lysosomes exposed to cysteine when compared with exposure to immobilized cysteine. Conversion of [3H]MTX + G1 to [3H]MTX, on the other hand, was observed only in samples exposed to free cysteine, accounting for the differential in the loss with time of the polyglutamate in the presence and absence of cysteine. Only trace levels of MTX were detected in the samples exposed to immobilized cysteine or not exposed to sulfhydryls at all.

It was found during the course of these experiments that exposure of lysosomes to 5 mM cysteine resulted in a small reduction in the fraction of intact lysosomes as determined from the measurement of latent β-hexosaminidase. Presumably this is the result of excessive cysteine accumulation leading to osmotic disruption of lysosomes, as suggested by the work of others (17). The results shown in Figs. 2 and 3, therefore, have been corrected for any loss of the intact lysosomal fraction during the experiment and the differences shown reflect to our best estimate the effect of this agent on intralysosomal hydrolysis. We also point out that substitution of cysteine with MET which has no effect on lysosomal integrity, generates results similar to those shown for the former in Figs. 2 and 3 (data not shown).

Hydrolysis of Methotrexate Polyglutamates in Permeabilized Lysosomes—Given the limitations posed by the lysosomal membrane for the study of enzyme-substrate interactions, the kinetics for MTXPG hydrolysis were examined in preparations made permeable by exposure to 0.1% Triton X-100. Separate experiments (data not shown) comparing sonication with detergent exposure as a means to disrupt lysosomes were used to establish that the presence of this concentration of Triton X-100 in the assay medium did not affect FPG hydrolyase activity, but acted only to eliminate the membrane barrier and allow direct access of substrate to the enzyme. In order to facilitate comparison between results obtained for the hydrolysis and transport steps, the data for enzyme activity were expressed as a function of β-hexosaminidase activity, in the same units as the transport step. Measurements of hydrolytic activity in lysosomal preparations exposed to 10 mM MET in the presence and absence of 0.1% Triton X-100 are
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given in Fig. 4. In each case, the level of hydrolytic activity was determined by the amount of MTX liberated in the lysosomal suspension as shown by HPLC analysis. At a concentration of 100 μM of MTX + G1, the rate of hydrolysis was 20-fold greater in lysosomes permeabilized by Triton X-100. Other measurements of activity made at various pH values in the presence of 0.1% Triton X-100 and 10 mM MET show (Fig. 5) that FPG hydrolase exhibits a broad pH optimum in the range of 4.5–6. Based on these data and on data from published reports (18, 19) measuring intralysosomal pH, subsequent enzyme assays were conducted at pH 5.5.

Additional information on the kinetics for MTXPG hydrolysis was obtained from concentration-response experiments measuring FPG hydrolase activity as a function of substrate concentration for MTX + G1, MTX + G2, MTX + G3, and MTX + G4. Rates of hydrolysis were determined from individual time point determinations made after incubation of lysosomes at 37 °C for either 15 or 20 min in the presence of 0.1% Triton X-100, 10 mM MET, and the appropriate substrate concentrations. Linearity of the time course for hydrolysis was established in separate experiments (data not shown) at 37 °C for up to 30 min, at various concentrations for each of the four substrates tested. The velocity versus substrate concentration and double-reciprocal plots prepared from these data are shown for the simplest polyglutamate, MTX + G1, in Fig. 6. FPG hydrolase activity obeyed Michaelis-Menten kinetics and exhibited a single saturable component for each of the four substrates. Double-reciprocal analysis of the concentration response data was used to determine kinetic constants for the hydrolysis of MTXPGs in permeabilized lysosomes. Apparent $K_m$ and $V_{max}$ values derived by this type of analysis are shown in Table I. $K_m$ values were in the low micromolar to high nanomolar range and exhibited an inverse relationship to polyglutamate chain length. In contrast, $V_{max}$ was relatively constant for each of the four substrates tested.

Also shown in Table I are the corresponding data for facilitated transport of MTX polyglutamates that was obtained from our recent publication (8). These studies provided values for $K_m$ and $V_{max}$ of influx of [3H]MTX + G1 and values for $K_i$ for MTX + G1, MTX + G2, MTX + G3, and MTX + G4 as inhibitors of [3H]MTX + G1 influx. Comparison of both sets of data given in the table reveal much higher saturability (100–200-fold) of the hydrolytic process than of transport of MTXPGs in lysosomes, but similar capacities, under the conditions of these experiments. A similar relationship is also shown for the values of $V_{max}/K_m$ calculated from the hydrolysis and transport data obtained in each study.

**DISCUSSION**

Based on the data presented in this report and that available in our earlier publication (8), we have constructed a simple model depicting fundamental events regulating free pools of MTXPGs, as well as the naturally occurring folate polyglutamates, in the cell. A schematic of this model is shown in Fig. 7. It illustrates a two-step process reflecting the compartmentalization of the degradative process in lysosomes, well removed from the synthetic process occurring in the cytoplasm. It also emphasizes the key role played by the
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Kinetic parameters for lysosomal transport and hydrolysis of MTXPGs

Measurement of initial influx and hydrolysis of the various MTX polyglutamates was described in detail in the text. The data shown are averages of three to five experiments done on separate days. Standard error of the mean = ±15%. hex, hexosaminidase.

| Substrate | Initial influx* | FPG hydrolase activity |
|-----------|----------------|------------------------|
|           | $K_a$ (μM) | $V_{max}$ (pmol/min/hex) | $K_i$ (μM) | $V_{max}/K_a$ | $V_{max}/K_i$ |
| MTX + G1  | 346 ± 39  | 2.8 ± 0.3 | 334 ± 19  | 0.008 | 3.07 ± 2.5 | 3.50 ± 0.09 | 1.14 |
| MTX + G2  | 201 ± 16  | 0.09 ± 0.09 | 3.68 ± 0.15 | 3.90 |
| MTX + G3  | 106 ± 13  | 0.47 ± 0.04 | 3.40 ± 0.04 | 7.47 |
| MTX + G4  | 42 ± 8    | 0.24 ± 0.03 | 3.47 ± 0.08 | 14.63 |

* These data were extracted from results published in Ref. 8.

Fig. 7. Schematic diagram showing the dynamic interaction between synthesis and degradation of MTXPGs in the different cellular compartments and the mediation of these two events by transport across the lysosomal membrane.

The transport step at the level of the lysosomal membrane, as shown by various lines of evidence. This step and the intralysosomal availability of cysteine via a well characterized (14–17) transport system is limiting to the turnover of polyglutamylated folates and antifolates in the cell. Finally, we also indicate, that although mediated exit of cysteine has also been documented by the work of others (14–17), the precise manner by which MTX is rapidly extruded from the lysosome, which was documented during these studies, remains unknown. Since MTX is a negatively charged bivalent anion, which would not be expected to diffuse passively through the lysosomal membrane, it is likely that a specific transport mechanism is also involved in this case as well. The fate of the glutamyl peptide is also unknown, but it is assumed to exit intact or following degradation to shorter peptides and/or glutamate.

Fig. 7. Schematic diagram showing the dynamic interaction between synthesis and degradation of MTXPGs in the different cellular compartments and the mediation of these two events by transport across the lysosomal membrane.

Comparison of the kinetics for transport and hydrolysis demonstrates the large differential that exists in the efficiency of lysosomes to carry out these sequential steps. For instance, $V_{max}/K_a$ ratios are 140-fold greater for degradation of the simplest polyglutamate, MTX + G1, and there is a 260-fold average differential for the longer chain polyglutamates tested, namely MTX + G2, MTX + G3, and MTX + G4. These calculations were made from data directly measuring transport in intact lysosomes and the hydrolysis of the polyglutamate in permeabilized lysosomes at the optimum, or near optimum, conditions for each process. Interestingly, this differential is largely reflected in the saturability ($K_a$ or $K_i$) for each process, which is in the mid-micromolar range for transport and low-micromolar to high-nanomolar range for hydrolysis. In contrast, values for $V_{max}$ were similar for the transport and hydrolysis steps, respectively. It may be inferred from this comparison that the limiting role of permeation in regard to MTX polyglutamate turnover across the lysosomal membrane reflects the low-affinity kinetics of the substrate for the transporter and the high-affinity kinetics of the internalized substrate for the enzyme system ultimately responsible for hydrolysis. This inference was confirmed in practice by the results from experiments (Fig. 1) showing that degradation of MTXPGs in intact lysosomes is limited by their intralysosomal accumulation, where FPG hydrolase is fully activated by reduced sulfhydryls. These results are in sharp contrast to the much higher rate of hydrolysis of this substrate in the presence of sulfhydryls that is seen in permeabilized lysosomes. Further evidence was obtained from experiments where reduced sulfhydryls were added to lysosomes that had been previously loaded with substrate (Fig. 3). These results show that overall levels of accumulated substrate decreased more rapidly in lysosomes exposed to reduced sulfhydryls, but not to nonpermeable reduced sulfhydryls or in controls, and that this decrease was accompanied by a proportional increase in substrate hydrolysis.

The experiments demonstrating activation of hydrolysis by reduced sulfhydryls are in agreement with published reports (5–7, 13) studying biochemical properties of purified lysosomal FPG hydrolase. These studies employed MET to activate enzymatic activity in vitro. However, the work of Lloyd (16), and later Pisoni et al. (17), provides a strong argument to suggest that cysteine may be the naturally occurring reduced sulfhydryl capable of participating in a variety of lysosomal reactions in the cell. This amino acid is effectively transported across the lysosomal membrane by a specific transport system (17), and the resulting oxidized product, cystine, is rapidly released from the lysosome to be regenerated in the cytoplasm back to cysteine (17). Glutathione, another potential candidate for this role, has been shown not to permeate lysosomes (14), but does provide the reductive power necessary to regenerate cystine to cysteine in the cytosol (17). An unexpected finding was the high concentration of cysteine required (Fig. 2B) to activate FPG hydrolase. Although cellular cysteine concentration has been reported (20) to be in the range of 10–100 μM, concentrations of 100 μM and above were necessary to achieve significant activation of FPG hydrolase under the conditions of these experiments. This apparent discrepancy, however, may be reconciled by the work of Pisoni et al. (17) who have shown that cellular cysteine is largely sequestered in lysosomes, which comprise approximately 4% of the intracellular volume of normal human fibroblasts.

We also considered the relevance of these findings to studies on folic acid and folic acid polyglutamate accumulation and persistence in intact tumor cells. Turnover of MTX polyglutamates has been shown to occur (1–4, 10, 21–23) during exposure of tumor cells to $^{3}H$MTX both in vitro and in vivo. However, turnover of these polyglutamates is much more rapid in vivo (1–4, 10, 21–23). Also, direct measurement of hydrolysis of MTX + G1 following preloading (22, 24) of in
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Vivo derived tumor cells with this polyglutamate has been shown to be rapid. This can be explained by results (10) showing that levels of FPG hydrolase activity in tumor cells are consistently higher in vivo than in the same cells maintained in culture. Since the lysosomal preparations used in these studies were derived from S180 cells of animal origin, the results presented here should probably be considered in the context of the data derived during studies of MTX polyglutamate turnover in vivo. To this end, our results and those of the prior (21–23) in vivo studies suggest that folyl and anti-folylpolyglutamate synthesis and turnover are extremely dynamic processes and that lysosomal-mediated hydrolysis of polyglutamates may be required to regulate their levels within the cell in the absence of some sort of regulation at the level of polyglutamate synthesis.

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