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Submitochondrial localization of the mitochondrial isoform of folylpolyglutamate synthetase in CCRF-CEM human T-lymphoblastic leukemia cells

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Abstract

Earlier studies from this laboratory showed that human folylpolyglutamate synthetase (FPGS) exists as cytosolic and mitochondrial (mFPGS) isoforms. Localization of mFPGS within mitochondria may help elucidate how the enzyme functions to maintain the mitochondrial folate pool. A human T-lymphoblastic leukemia CCRF-CEM cell lysate was fractionated by differential centrifugation into cytosolic and mitochondrial fractions. Activity assays for cytosol-and mitochondria-specific enzymes verified the purity and integrity of the fractions. Mitochondria were subfractionated with increasing concentrations of digitonin to successively extract the four submitochondrial compartments. Western analyses of the fractions using protein markers specific for each compartment suggest that mFPGS is distributed in the matrix and/or inner membrane compartments. Further support for an interaction of mFPGS with the inner mitochondrial membrane is provided by localization of about half of the mFPGS in the mitochondrial membrane fraction obtained by freeze–thaw of intact mitochondria; the remaining mFPGS is located in the soluble fraction. Resistance of about half of the mFPGS in whole mitochondria to alkaline carbonate extraction suggests that its interaction with the inner membrane is more similar to an integral, than a peripheral, membrane protein. The data suggest that human mFPGS is at least in part strongly associated with the inner mitochondrial membrane.

Keywords: Folylpolyglutamate synthetase; Polyglutamate; Mitochondria; Folate; Leukemia; CCRF-CEM

1. Introduction

Folylpolyglutamate synthetase (FPGS) catalyzes the ATP-dependent addition of glutamate residues to the γ-carboxyl of folates to synthesize folylpoly(γ-glutamates) [1], which are the predominant folates in cells. Mammalian cells utilize folylpolyglutamates as more efficient substrates for most of the folate-dependent enzymes that are involved in the one-carbon transfer reactions involved in purine, pyrimidine and amino acid synthesis. Folylpolyglutamates also aid in intracellular retention of folates at concentrations much higher than that in the extracellular milieu. Both of these functions are required for cell viability and thus FPGS is an essential enzyme [2]. FPGS exists as cytosolic (cFPGS) and mitochondrial (mFPGS) isoforms [3] that are transcribed from one nuclear gene as two classes of mRNAs [4–6]. One class codes only for cFPGS, while the second class has two different in-frame translational start sites, one of which also encodes cFPGS, while the

Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; cFPGS, cytosolic FPGS; COX IV, cytochrome *c* oxidase subunit IV (EC 1.9.3.1); CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-[5′-chloro]tricyclo[3.3.1.1^{3,7}]decan}-4-yl]phenyl phosphate; cytC, cytochrome *c* (SwissProt Q6NUR2); EDTA, ethylenediaminetetraacetic acid; FPGS, folylpolyglutamate synthetase (EC 6.3.2.17); GDH, glutamate dehydrogenase (EC 1.4.1.3); HEPES, 1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-free acid; H-medium, 70 mM sucrose, 220 mM mannitol, 2 mM HEPES adjusted to pH 7.4 with KOH and made up to 0.16 mg/ml in benzamidine-HCl, 0.5 mM in Pefabloc, and 0.5 mg/ml in protease-free BSA; IM, inner mitochondrial membrane; IMS, intermembrane space; LDH, lactate dehydrogenase (EC 1.1.1.27); mFPGS, mitochondrial FPGS; MnSOD, manganese superoxide dismutase (EC 1.15.1.1); NADH, nicotinamide adenine dinucleotide (reduced); OM, outer mitochondrial membrane; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PNS, postnuclear supernatant; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; VDAC, voltage-dependent anion channel protein (SwissProt P21796)

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upstream second start site encodes a protein with an additional 43 amino acids at the N-terminal that have the characteristics of a mitochondrial signal sequence. Studies of the regulation of transcription and translation of these mRNAs are ongoing. cFPGS and mFPGS maintain folylpolyglutamate pools in the cytosol and mitochondria, respectively. Studies by Lin and Shane [7] have shown, however, that mitochondrial folylpolyglutamates may exit to the cytosol at a slow rate and allow for cell viability in the absence of cFPGS. Thus, mFPGS may play a critical role in maintaining cellular folate homeostasis.

We have undertaken to determine the submitochondrial location of mFPGS because its location may influence its physiological function. Digitonin fractionation [8] of isolated mitochondria was used to sequentially release the protein contents of the outer membrane, intermembrane space, inner membrane, and matrix. Because the results indicated that up to 50% of the mFPGS might be associated with the inner membrane, we used freeze–thaw [9] and alkaline carbonate extraction procedures [10] to verify its submitochondrial location and to study the nature of the association of mFPGS with the inner membrane.

2. Materials and methods

2.1. Materials

Digitonin (ultrapure) and benzamidine–HCl were purchased from Calbiochem, La Jolla, CA. α-Ketoglutaric acid (Na_2 salt), Na-pyruvate, ADP, and Pefabloc SC were from Sigma Chemical Co. (St. Louis, MO), Nutritional Biochemicals Corp. (Cleveland, OH), USB Corp. (Cleveland, OH), and Centerchem Inc. (Stamford, CT), respectively. NADH (Na_2 salt) and CSPD were from Roche Biochemicals (Indianapolis, IN). Immobilon-P polyvinylidene difluoride (PVDF, Millipore) membrane was obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were of the highest grade available.

2.2. Protein determination

Protein concentration was determined by the Bradford assay [11] using a BioRad (Hercules, CA) protein assay kit. All readings were corrected for interference from buffer components.

2.3. Cell culture

The CCRF-CEM human T-lymphoblastic cell line was cultured as described [12]; it was periodically screened for mycoplasma contamination using Stratagene Mycoplasma Plus™ PCR primers (La Jolla, CA) and was confirmed negative. Cell numbers were quantitated using a Coulter model Z1 counter (Beckman Instruments; Fullerton, CA) with a 140-μ aperture.

2.4. Isolation and characterization of human cytosolic and mitochondrial fractions

Subcellular fractionation of CCRF-CEM extracts into cytosolic and mitochondrial fractions was performed essentially as described [3]. Postnuclear supernatant (PNS), cytosol and mitochondrial fractions were assayed for activity of the cytosolic enzyme lactate dehydrogenase (LDH) [13] and mitochondrial matrix enzyme glutamate dehydrogenase (GDH) [14] to verify purity, yield and integrity of the mitochondrial fraction. Assays were performed in quartz cuvettes at 340 nm using a Gilford 240 spectrophotometer over a 3- to 4-min time period at 25 °C. All values reported are the average of two sample dilutions assayed in duplicate to verify enzyme linearity. LDH assays (1 ml) contained 120 mM Tris–HCl (pH 7.3), 0.2 mM Na_2-NADH and 250 mM sucrose and were initiated by addition of Na-pyruvate to a final concentration of 0.9 mM. GDH assays (1 ml) contained 100 mM imidazole (pH 7.9), 220 mM ammonium acetate, 0.2 mM Na_2-NADH, 0.9 mM Na_2-EDTA (pH 7.5), 1.7 mM Na_2-ADP and 250 mM sucrose and were initiated by addition of α-ketoglutarate to a final concentration of 14 mM. GDH activity was measured in the presence and absence of 0.1% (v/v) Triton X-100 to assess mitochondrial integrity (latency). Latency was calculated as a percent ratio of the difference in GDH activity with and without Triton X-100 to the GDH activity with Triton X-100. Mixing of the cytosolic fraction with the mitochondrial fraction yielded 97% and 100% of the expected LDH and GDH activity, respectively ($n=2$).

2.5. Submitochondrial fractionation with digitonin

All procedures were performed at 4 °C. The mitochondrial pellet (above) was suspended in H-medium (70 mM sucrose, 220 mM mannitol, and 2 mM HEPES, all adjusted to pH 7.4 with KOH, and made up to 0.16 mg/ml in benzamidine–HCl, 0.5 mM in Pefabloc, and 0.5 mg/ml in protease-free BSA) [15] to a final protein concentration of 10 mg/ml. The mitochondrial suspension was divided into 8 fractions and digitonin [16] (from a stock of 1.6% (w/v) in H-medium) was added to a final concentration of 0–0.7% (w/v) in increments of 0.1% (w/v) [8]. Samples were incubated for exactly 1 min on ice, quickly diluted with 4 volumes of ice-cold H-medium, and centrifuged at 48,000×g for 20 min at 4 °C in a Beckman SW-55Ti swinging bucket rotor. The supernatant was saved separately. Pellets were suspended in 1/4th supernatant volume of H-medium and vortexed and sonicated for 30 s in a Branson-1200 sonic water bath (Branson, Danbury, CT) at the maximum setting to homogenize the suspension. Pellet suspensions were assayed by quantitative Western analysis (below) for release of marker proteins for each mitochondrial subcompartment and for mFPGS.

2.6. Freeze–thaw fractionation of mitochondria

The mitochondrial suspension in isotonic buffer [4] from the subcellular fractionation procedure was centrifuged at 10,000×g_{max} for 15 min at 4 °C in a Beckman-Coulter microfuge 22 R and the supernatant was discarded. The mitochondrial pellet was suspended to its original volume in 50 mM Tris–HCl, pH 7.5, 0.5 mM Na_2-EDTA, pH 7.5, 0.5 mM Pefabloc, and 1 mM benzamidine and lysed by freezing for 5 min in liquid N_2 and then thawing rapidly by warming between the fingertips just until the last ice melted. The freeze–thaw was performed 4 times. The suspension was centrifuged at 144,000×g_{av} at 4 °C for 1 h in a Beckman TLA100 tabletop micro-ultracentrifuge using the TLA100.3 rotor. The supernatant was removed and the pellet was resuspended in the original volume of the same buffer, freeze–thawed four times as described above, and centrifuged as above. Both supernatants were pooled and used as the soluble fraction. The pellet was resuspended in isotonic buffer and used as the membrane fraction. Soluble and membrane fractions were assayed by quantitative Western analysis (below) for the presence of mFPGS and protein markers.

2.7. Alkaline carbonate extraction of intact mitochondria

Alkaline carbonate extraction of intact mitochondria was performed as described [10]. Briefly, a mitochondrial suspension in isotonic buffer was treated with an equal volume of ice-cold 200 mM Na_2CO_3, pH 11.5 containing 0.5 mM Pefabloc and mixed by tapping and vortexing to a final concentration of 100 mM Na_2CO_3. Mitochondria similarly treated with an equal volume of 266 mM NaCl containing 0.5 mM Pefabloc to a final concentration of 133 mM NaCl (experimentally determined to be equivalent in ionic strength to 100 mM Na_2CO_3) were maintained as a control. Both treatments were incubated on ice for 30 min. The samples were centrifuged at 144,000×g_{av} at 4°C for 1 h in a Beckman TLA100 tabletop micro-ultracentrifuge using the TLA100.3 rotor. The pellets were resuspended to their original volume (before Na_2CO_3 addition) in 100 mM Na_2CO_3, pH 11.5, 0.5 mM Pefabloc or 133 mM NaCl, 0.5 mM Pefabloc, respectively, incubated on ice again for 30 min, and centrifuged as above. Pellets were suspended in isotonic buffer to yield the membrane fraction, while the supernatants from both the extractions were