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MitoTracker Green labeling of mitochondrial proteins and their subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection

Andrew D. Presley, Kathryn M. Fuller, Edgar A. Arriaga*

Department of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis, MN 55455, USA

Abstract

MitoTracker Green (MTG) is a mitochondrial-selective fluorescent label commonly used in confocal microscopy and flow cytometry. It is expected that this dye selectively accumulates in the mitochondrial matrix where it covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues. Here we demonstrate that MTG can be used as a protein labeling reagent that is compatible with a subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF). Although the MTG-labeled proteins and MTG do not seem to electrophoretically separate, an enhancement in fluorescence intensity of the product indicates that only proteins with free thiol groups are capable of reacting with MTG. In addition we propose that MTG is a partially selective label towards some mitochondrial proteins. This selectivity stems from the high MTG concentration in the mitochondrial matrix that favors alkylation of the available thiol groups in this subcellular compartment. To that effect we treated mitochondria-enriched fractions that had been prepared by differential centrifugation of an NS-1 cell lysate. This fraction was solubilized with an SDS-containing buffer and analyzed by CE–LIF. The presence of a band with fluorescence stronger than MTG alone also indicated the presence of an MTG–protein product. Confirming that MTG is labeling mitochondrial proteins was done by treating the solubilized mitochondrial fraction with 5-furoylquinoline-3-carboxaldehyde (FQ), a fluorogenic reagent that reacts with primary amino groups, and analysis by CE–LIF using two separate detection channels: 520 nm for MTG-labeled species and 635 nm for FQ-labeled species. In addition, these results indicate that MTG labels only a subset of proteins in the mitochondria-enriched fraction.

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1. Introduction

The analysis of the proteins found in a given organelle type facilitates defining the connection between a protein's subcellular localization and its function. For instance, by looking at the mitochondrial proteome it would be easier to study the protein complexes that participate in the oxidative phosphorylation process than by studying the complete proteome. To this effect, subcellular fractionation by centrifugation [1] followed by 2D-gel electrophoresis has proven to be effective in detecting proteins that are found in the same organelle fraction [2–5]. Unfortunately, the entire process from sample preparation to running and developing the 2D-gel is labor-

*Corresponding author. Tel.: +1-612-624-8024; fax: +1-612-626-7541.

E-mail address: arriaga@chem.umn.edu (E.A. Arriaga).

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intensive, time-consuming, and limited by the purity of the subcellular isolate [2,6,7].

Capillary electrophoresis (CE) with UV absorbance detection or laser-induced fluorescence detection (LIF) is another separation technique useful in the analysis of proteins [8,9]. This technique is compatible with low sample volumes and typically separations are more rapid than with 2D-gel electrophoresis [10]. Furthermore, the use of fluorescent or fluorogenic labeling reagents further enhances detection sensitivity [11]. In particular, the use of fluorogenic reagents which fluoresce only after reaction with an analyte has been shown to result in lower background signals and reduce the limits of detection to the picomolar range [12–14]. CE–LIF analysis of fluorescently-labeled proteins extracted from cultured cells has been reported previously but it lacks the resolving power to obtain a comprehensive proteome profile [15]. A subcellular fractionation step prior to CE analysis would help to obtain a protein profile that is representative of a specific subcellular component. Enzyme profiling from subcellular fractions is commonplace [16] and we have reported the application of CE to analyze drug metabolites from various subcellular fractions [17].

Further subcellular definition of a protein profile could be attained if protein labeling was organelle-selective. Lin et al. have used 4-(iodobutyl)-triphenylphosphonium that accumulates in the mitochondria due to its membrane potential and the high pH in the matrix [18]. This compound mainly reacts with thiol groups of proteins found in the matrix and the inner membrane surface facing the matrix. MitoTracker dyes (Molecular Probes, Eugene, OR, USA) that are used in flow cytometry [19,20] and confocal microscopy [20–23] to identify mitochondria, have characteristics that would make them good candidates for subcellular-selective protein labels. These dyes have two chloromethyl moieties that upon accumulation in the mitochondrial matrix may react with thiol groups of cysteine residues, thereby becoming covalently bound to mitochondrial protein [21,24]. Considering their fluorescent properties, their selectivity towards thiol groups, and their preferential accumulation in mitochondria, MitoTracker dyes are good candidates for fluorescently labeling mitochondrial proteins that then can be analyzed by CE–LIF. Previously we used MitoTracker Green (MTG) to detect individual mitochondria by CE–LIF and observed that the number of mitochondrial events was in agreement with the expected relative abundance of enzymatic mitochondrial markers in several subcellular fractions [25]. Although we suggested that the MTG fluorescence intensity for each detected event is related to the protein abundance in the individual mitochondrion, we were unable to confirm if indeed MTG was covalently bound to protein or had been accumulated in these organelles due to the presence of a membrane potential or the high pH in the matrix.

Here we report that CE–LIF analysis reveals that MTG indeed labels proteins either in the form of pure standards in solution or as constituents of mitochondria-enriched fractions from NS1 mouse hybridoma cells. These findings provide validation that MTG fluorescence in individual mitochondria is due to covalent protein labeling. Furthermore, double labeling with MTG and 5-furoylquinoline-3-carboxaldehyde (FQ) suggests that MTG labels only a subset of proteins found in the mitochondrial fraction. The preferential labeling of some proteins may result from their presence in the matrix or the surface of the inner mitochondrial membrane facing the matrix where MTG is expected to reach concentrations 300-fold higher than in the medium surrounding the mitochondrion.

2. Experimental

2.1. Reagents

D(+)-Mannitol was from Acros Organics (Morris Plains, NJ, USA). Ethylenediaminetetraacetic acid (EDTA) was from Avocado Research Chemicals (Heysham, Lancashire, UK). *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), D-mannitol, Dulbecco's Modified Eagle's Medium, calf serum, aprotinin from bovine lung, and phospholipase D (type IV from cabbage) were purchased from Sigma (St. Louis, MO, USA). Sodium tetraborate and sucrose were from EM Science (Gibbstown, NJ, USA). Dimethyl sulfoxide (DMSO) and digitonin were from Aldrich (Milwaukee, WI, USA). Sodium dodecylsulfate (SDS) and sodium hydroxide

were purchased from JT Baker (Phillipsburg, NJ, USA). Hydrochloric acid (37%) and potassium hydroxide were from Mallinckrodt (Paris, KY, USA). MitoTracker Green FM (MTG), 5-furoylquinoline-3-carboxaldehyde (FQ), and potassium cyanide were purchased from Molecular Probes (Eugene, OR, USA).

The mitochondrial isolation buffer, ''Buffer M'', contained 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES, and 5 m*M* EDTA; pH was adjusted to 7.3 with potassium hydroxide. The CE buffer, ''BS buffer'', contained 10 m*M* borate and 20 m*M* SDS; pH was adjusted to 9.4 with potassium hydroxide and the solution was degassed under vacuum when required. Aqueous 1 *M* solutions of NaOH and HCl were used to condition the capillary between runs. All aqueous solutions and buffers were made with Milli-Q deionized water and filtered prior to use (0.22 μm). A working digitonin solution (10 mg/mL in Buffer M) was prepared from a stock solution (100 mg/mL in DMSO) immediately before use. Labeling solutions were prepared from an MTG stock solution (1 m*M* in DMSO) and dried FQ aliquots (100 nmol, prepared as previously described [13]) immediately before use. Solutions of aprotinin and phospholipase D with protein concentrations of 4.0 mg/ml (620 μ*M*) and 2.0 mg/ml (22 μ*M*), respectively, were prepared in water. The aprotinin solution was diluted to 30 μ*M* in water prior to usage. For labeling of these standard protein solutions a 20 μ*M* MTG solution in Buffer M was prepared immediately prior to usage.

2.2. MTG-labeled protein standards

Since phospholipase D contains eight cysteine residues whereas aprotinin contains only six, samples that contained 1 μ*M* MTG were prepared such that there was a 1:1 ratio of MTG to cysteine residues. Thus, the aprotinin concentration was 0.17 μ*M* and the phospholipase solution was 0.12 μ*M*. All samples were prepared in buffer M and were incubated in the dark at 37 °C for 15 min and immediately diluted three-fold in BS buffer. Controls consisting of unreacted MTG and unlabeled protein samples as well as solutions of MTG and protein that were boiled for 10 min (following incubation and prior to the addition of SDS) to hasten denaturation and exhaustively label the proteins were also prepared.

2.3. Preparation and labeling of subcellular fractions

NS1 mouse hybridoma cells were cultured at 37 °C and 5% CO_2 in 90% Dulbecco's Modified Eagle's medium, 10% calf serum. Cells were harvested in the late log phase and washed twice with cold Buffer M. The cells were resuspended in Buffer M at a concentration of 10^7 cells/mL. To an Eppendorf tube containing 1000 μl of cell suspension, 3.5 μl of 10 mg/ml digitonin were added and then incubated on ice for 5 min. The tube was then placed in an ice-cold nitrogen cavitation bomb (Model 4639, Parr Instrument, Moline, IL, USA), pressurized to 700 p.s.i. with high-purity nitrogen, and equilibrated on ice for 20 min. The nitrogen was quickly released from the bomb to disrupt the cells. Disruption (~80%) was monitored by light microscopy.

After cell disruption, mitochondria were isolated by differential centrifugation as described elsewhere [26]. Briefly, the cell lysate was spun for 10 min at 1000 *g* to clear whole cells and nuclei. The separated supernatant was spun a second time to further eliminate whole cells and nuclei. The supernatant was then spun at 10 000 *g* for 10 min to obtain a pellet enriched in mitochondria; in this pellet the main contaminants are lysosomes but traces of other subcellular contaminants are also expected. On the other hand, the supernatant from spinning the preparation at 10 000 *g* (cytosolic components) is expected to contain microsomes resulting from disruption of membranous organelles. The mitochondria-enriched pellet was resuspended in Buffer M and spun a second time to further eliminate lighter components. The resulting pellet was then resuspended in MTG (1 μ*M* in Buffer M) and incubated at 37 °C for 15 min. The preparation was spun again for 10 min at 10 000 *g*, and the MTG-containing supernatant was removed. Finally, the mitochondria-enriched fraction was solubilized in 40 μL of 10 m*M* borate–1% (w/v) SDS, pH 9.4. The cellular supernatant containing cytosolic and microsomal components was labeled with 1 μ*M* MTG in Buffer M at 37 °C as a

control for cytosolic components. Samples were kept on ice before analysis by CE–LIF.

2.4. FQ-labeling of mitochondria-enriched fraction

For dual labeling experiments, the MTG-labeled mitochondrial-enriched fraction was also derivatized with FQ according to a procedure used for a picomolar protein assay by CE–LIF [13]. Briefly, the solubilized mitochondria-enriched pellet (9 μL) was added to a vial containing 100 nmol of dried FQ, followed by the addition of 1 μL of 25 m*M* potassium cyanide in methanol (10 m*M* FQ). The proteins were incubated for 10 min at 65 °C. In order to investigate the progress of the FQ-labeling reaction, the temperature was set at 40 °C and 2-μL aliquots were removed at 15, 30, and 50 min.

2.5. CE separation of MTG and FQ-labeled samples

Separations were carried out using the P/ACE MDQ Glycoprotein System (Beckman Coulter, Fullerton, CA, USA). Protein samples were separated in 50 μm (I.D.) fused-silica capillaries (Polymicro Technologies, Pheonix, AZ, USA). The capillary detection window was prepared by burning the capillary polyimide outer coating with a flame about 10 cm from the detection end; the exact position of the detection window is listed in each figure caption. The 488-nm line of an argon ion laser was used for excitation. Dual-channel LIF detection was performed with a 530±25.0 nm band-pass filter (144940, Beckman) for MTG fluorescence and a 635±27.5 nm band-pass filter (635DF55, Omega Optical, Brattleboro, VT, USA) for FQ-related fluorescence.

Before beginning the CE analysis of the protein standards the capillary was flushed for 10 min at 20 p.s.i. with each of the following solutions: H_2O, 1 *M* HCl; H_2O; 1 *M* NaOH; and H_2O; All samples were injected hydrodynamically for 5 s at 1 p.s.i. or at 100 V/cm. For those samples containing cellular material, the capillary was flushed for 30 min at 20 p.s.i. with each of the following solutions: 1% HCl; 1 *M* NaOH; H_2O; and BS buffer. These samples were diluted in BS buffer and injected electrokinetically for 5 s at 100 V/cm, positive polarity. Unless otherwise noted, all samples were separated in BS running buffer at 400 V/cm, positive polarity. Between runs the capillary was flushed for 2 min at 20 p.s.i. with each of the following solutions H_2O, 1 *M* HCl; H_2O; 1 *M* NaOH; H_2O; and BS buffer. Data were collected at 16 Hz and exported to Igor Pro (Wavemetrics, Lake Oswego, OR, USA) for analysis.

2.6. Fluorescence emission spectra of MTG-labeled samples

Mitochondria were prepared by differential centrifugation as described above. The mitochondria-enriched fraction was suspended in 70 μL of Buffer M containing 1 μ*M* MTG and incubated at 37 °C for 15 min. Fluorescence emission spectra were collected for (1) 1 μ*M* MTG in Buffer M; (2) the mitochondria suspension containing MTG; and (3) the mitochondria after they had been pelleted from the MTG-containing solution, washed in Buffer M (as described above), and resuspended in 70 μL of Buffer M; (4) MTG-labeled mitochondria-enriched fractions after solubilization with 1% (w/v) SDS. The fluorescence emission spectrum for unlabeled mitochondria in Buffer M was collected as a control for mitochondrial autofluorescence. All spectra were produced with excitation at 488 nm and collected with an FP-7200 Spectrofluorometer (Jasco, Easton, MD, USA) using a quartz 50-μL spectrophotometer cell (Starna, Atascadero, CA, USA).

3. Results and discussion

One of MTG's primary attributes is that it accumulates in the mitochondrion due to the membrane potential, thus low concentrations can be used for labeling of this organelle while maintaining a low fluorescence background [27]. Given that the mitochondrial membrane potential ($\Delta\psi_m$) is about −150 mV [28], the Nernst equation:

$$\Delta\psi_m = \frac{-RT}{2F} \ln\frac{[\mathrm{MTG}]_{in}}{[\mathrm{MTG}]_{out}}$$

can be used to estimate the dye concentration in the mitochondrial membrane matrix $[\mathrm{MTG}]_{in}$ based on the dye concentration outside the inner mitochondrial

membrane $[MTG]_{out}$. If T is set to 37 °C (310 K); $[MTG]_{out}$ is set to 1 μ*M*, the concentration during labeling; and replacing the value of F (Faraday's constant), the corresponding value of $[MTG]_{in}$ is equal to 300 μ*M*. Therefore, the concentration of MTG in the mitochondrial matrix driven by a Nernstian behavior will be 300 times greater than in any of the other subcellular compartments. This 300-fold increase in MTG concentration ought to favor the selective labeling of mitochondrial proteins found in the matrix or the inner membrane surface facing the matrix over other proteins found elsewhere.

Bulk experiments confirmed that MTG not only accumulates in mitochondria but in addition that MTG shows a fluorescence enhancement upon accumulation in these organelles. Fluorescence intensity was 40-fold higher in the MTG emission spectra of 1 μ*M* MTG in buffer M in the presence and absence of mitochondria (Fig. 1D and A, respectively). When the organelles were pelleted and washed twice in Buffer M, the fluorescence intensity decreased by 50% (Fig. 1B), likely resulting from a loss of MTG not found in mitochondria or that leaked during the washing procedure.

The two MTG chloromethyl moieties (Fig. 1E) are believed to react with free thiol groups of cysteine residues in mitochondrial proteins, forming a stable thioether conjugate that is retained by the organelles

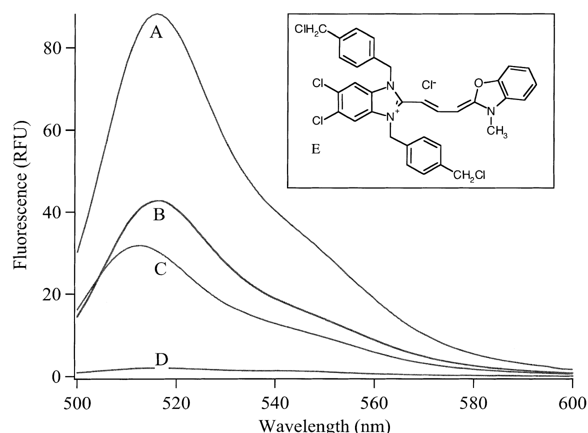

Fig. 1. Fluorescence emission spectra of MTG samples. (A) Mitochondria-enriched fraction incubated with 1 μ*M* MTG. (B) MTG-labeled mitochondria washed and suspended in Buffer M. (C) MTG-labeled mitochondria solubilized in BS buffer. (D) 1 μ*M* MTG in Buffer M. (E) MTG structure.

through fixing procedures and other biochemical techniques [24]. Similarly, in this report the MTG-labeled mitochondria-enriched fraction maintained 75% of its fluorescence intensity after solubilization in BS buffer (Fig. 1C). However, it is not possible to ascertain if the MTG remains associated with mitochondrial components. The emission spectrum for the SDS-solubilized sample also shows a slight blue shift in the MTG peak location, a possible result of interaction between SDS and the dye–protein conjugate. Taken as a whole, the spectrofluorimetry experiment indicates that accumulation of MTG in mitochondria results in enhanced fluorescence that is maintained after the organelles are solubilized in SDS. This is consistent with the hypothesis that after being concentrated by the mitochondrial membrane potential, MTG is thought to alkylate the free thiol groups of mitochondrial proteins. On the other hand, it does not provide direct evidence that MTG and proteins are covalently bound. As discussed below, CE–LIF provides more direct evidence of the existence of stable MTG–protein species.

3.1. Analysis of MTG-labeled protein standards by CE–LIF

Experiments using protein standards were designed to determine whether MTG can also label free proteins, thus providing insight into the true specificity of this mitochondrial probe. Aprotinin, a secreted trypsin inhibitor isolated from bovine tissues, contains six cysteine residues and three disulfide bridges [29]. Cabbage phospholipase D contains eight cysteine residues which have been shown to be free [30].

As can be seen in Fig. 2 when 1 μ*M* MTG is added to the phospholipase D in a 1:1 MTG:cysteine group molar ratio, incubated at 37 °C for 15 min, and analyzed by CE–LIF, it is not possible to distinguish between the migration times for MTG (dashed line) and the predicted MTG–phospholipase D reaction product (solid line) (180 and 181 s, respectively). On the other hand, the corresponding areas are different (1.8×10^5 and 2.6×10^5 RFU-s, respectively). Similar to the increase in fluorescence emission spectra for the bulk experiments (Fig. 1), the large peak area resulting from phospholipase D incubated with MTG suggests that MTG is able to label proteins in

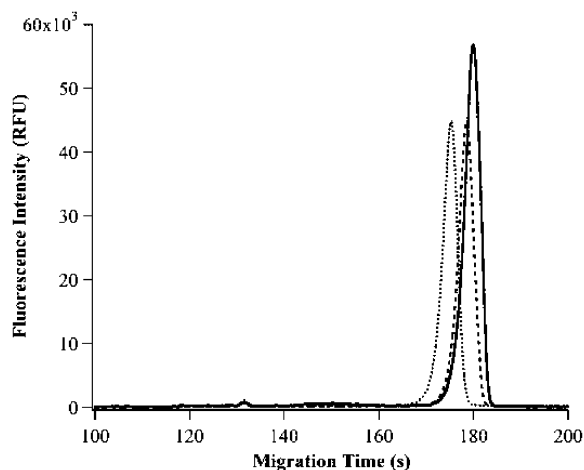

Fig. 2. CE–LIF analysis of aprotinin, phospholipase D and MTG standards. Phospholipase D labeled with MTG at final concentrations of 0.042 and 0.33 μ*M*, respectively (———); the trace has been offset −3.5 s to match the impurity peak at 129 s. Aprotinin treated with MTG at final concentrations of 0.056 and 0.33 μ*M* (· · ·); MTG at a final concentration of 0.33 μ*M* (- - -); the trace has been offset −2.5 s to match the impurity peak at 129 s. Capillary: fused silica, 31.2 cm×50 μm I.D.; detection window at 21.0 cm. Injections: 5 s, 1 p.s.i. Separations: 400 V/cm in BS Buffer. Solid trace indicates detection at 520±25 nm (Channel 1); dashed traced indicates detection at 635±27.5 nm (Channel 2).

solution as long as they have free thiol groups. Since phospholipase D has eight free thiol groups it is expected that it would be labeled to some extent even though it is not likely that all the thiol groups of this large protein are solvent (and thus MTG) accessible in the native form [31]. Further access to other thiol groups is possible if the protein is denatured, as confirmed when boiling an MTG–phospholipase D mixture prior to CE–LIF analysis. The corresponding fluorescence peak area was increased to 3.2×10^5 RFU-s (data not shown).

In contrast to phospholipase D, aprotinin was not expected to react with MTG since the thiol groups of aprotinin are not available for an alkylating reaction. As seen in Fig. 2 when 1 μ*M* MTG is added to the aprotinin in a 1:1 MTG:cysteine group molar ratio, incubated at 37 °C for 15 min, and analyzed by CE–LIF (dotted line), the corresponding peak area is basically the same as it was for MTG alone (dashed line) (1.9×10^5 and 1.8×10^5 RFU-s, respectively), suggesting that no reaction has occurred. On the contrary, the migration times for the MTG and the aprotinin reaction mixture are different (180 and 173 s, respectively). The variation in migration time cannot be presently explained but it is unlikely that an MTG–aprotinin product has been formed. In a separate experiment, we treated aprotinin with MTG and FQ, a fluorogenic reagent that labels primary amine groups. As expected from the presence of primary amino groups in the lysine residues of aprotinin, CE–LIF analysis indicated that this protein was successfully labeled with FQ and migrated 20 s earlier than the MTG peak (data not shown).

Although migration times cannot be used to unequivocally differentiate between free MTG and labeled proteins, the results shown in this subsection demonstrate that by monitoring intensity changes it is feasible to determine that proteins in solution (e.g. phospholipase D) can be labeled as long as they have a free thiol groups and 1 μ*M* MTG is used in the labeling reaction.

3.2. Is MTG a selective label for mitochondrial proteins?

We reported previously that MTG-treated mitochondria are detected individually by CE–LIF analysis when a buffer with pH and osmolarity compatible with intact organelles is used [25]. In that report, we assumed the fluorescence intensity for each detected event to be related with abundance of MTG-labeled proteins in the corresponding organelle. In order to further explore this assumption, here we solubilized several fractions with SDS and then analyzed them by CE–LIF. Fig. 3 shows several electropherograms (offset for clarity) resulting from injecting these solubilized fractions. Each electropherogram consists of two traces representing detection of MTG-related fluorescence at 530 nm (solid line) and FQ-related fluorescence at 635 nm (dashed line). The unlabeled mitochondria-enriched fraction does not (Fig. 3A) contain components that significantly auto-fluoresce at these two wavelengths. As expected, if 0.1 μ*M* MTG in BS buffer was injected, we detected a band in the 520 nm channel (5.2×10^5 RFU; RSD 3%, $n=5$) (Fig. 3B, solid line).

MTG Labeling of the supernatant left after separation of the mitochondria-enriched fraction at 10 000 *g* also resulted in labeled components when analyzed by CE–LIF (Fig. 3C, solid line). The

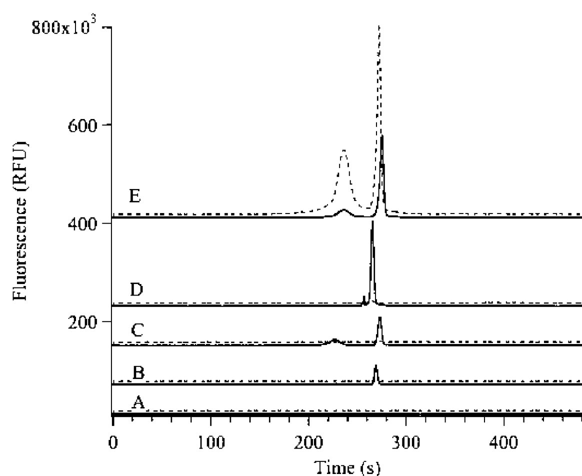

Fig. 3. CE–LIF analysis of MTG-labeled subcellular fractions. (A) Unlabeled mitochondria-enriched fraction; (B) 100 n*M* MTG; (C) MTG-labeled cytosolic fraction; (D) MTG-labeled mitochondria-enriched fraction; (E) MTG-labeled mitochondria-enriched fraction, derivatized with FQ. Capillary: fused silica, 40.4 cm × 50 μm I.D.; detection window at 30.0 cm. Injections: 5 s, 100 V/cm. Separations: 400 V/cm in BS Buffer. Solid trace indicates detection at 520±25 nm (Channel 1); dashed traced indicates detection at 635±27.5 nm (Channel 2). FQ labeling reaction was carried out at 65 °C.

electrophoretic profile for this subcellular fraction included a peak (t = 269 s) corresponding to unreacted MTG. It also showed a broad, low-intensity peak (t = 220–235 s) corresponding to labeled cellular material. The presence of the latter peak, in particular, indicates that it is possible for MTG to label, albeit to a lesser degree, proteins in organelles other than mitochondria such as ER Golgi microsomes, and other cytosolic components also found in this fraction. Supporting this possibility are our studies described in Fig. 2 that show that phospholipase D can be MTG-labeled and other studies that report on the distribution of MitoTracker dyes treated with membrane potential decouplers and sulfhydryl reagents [21,32,33]. These studies show that the loading and binding of MitoTracker dyes depends on the membrane potential and oxidation state of thiol groups in the organelles. In particular, the MTG analog chloromethyltetramethyl-rosamine (MitoTracker Orange) was found to accumulate in the nuclei of rat hepatoma cells [32]. Indeed, the cytosolic fraction may still contain residual mitochondrial contaminants that were not eliminated by differential centrifugation. However, as discussed below, the broad, low-intensity peak (t = 220–235 s) was not detected in the mitochondria-enriched fraction (Fig. 3D), suggesting that a different set of proteins are being labeled in this subcellular fraction. This statement is further supported by observing the electropherogram resulting from injecting the MTG-labeled mitochondria-enriched fraction that does not show this band but only a new band at t = 266±1 s (n = 5, Fig. 3D, solid line). It is worth noticing that if the band 220–235 was attributed to mitochondria, the corresponding band should be stronger in the mitochondria-enriched fraction (Fig. 3D, solid line). On the other hand, comparing the mitochondria-enriched fraction (Fig. 3D, solid line) with the 0.1 μ*M* MTG control (Fig. 3B), the MTG concentration expected if *all* MTG used in the labeling reaction was present in the injected sample, indicates fluorescence intensity enhancement in the mitochondria-enriched sample (1.62×10^5 RFU; RSD 23%, n = 5). This fluorescence enhancement is consistent with the fluorescence emission spectra shown in Fig. 1. Moreover, the potential contribution of unreacted MTG, not localized in mitochondria, to the measured peak height or area is expected to be negligible because the sample is washed twice with Buffer M after the MTG labeling reaction (see Experimental). Therefore, the signal corresponding to the mitochondria-enriched sample is expected to result from MTG that reacted with mitochondrial proteins during the labeling reaction or MTG that accumulated in mitochondria but was unable to react with cysteine residues.

As evidenced in Fig. 3B–D, MTG alone and MTG–protein complexes have similar migration times. Although the high concentration of SDS (20 m*M*) used in the running buffer (BS buffer) resulted in poor resolution when compared with lower SDS concentrations (e.g. 5 m*M*), it provided the best reproducibility in the migration time of the band observed in mitochondria-enriched fractions (RSD ≈0.4%; n = 6) (Fig. 3D). In the presence of such a concentration of SDS, the electrophoretic mobility differences among MTG-labeled proteins and MTG alone are masked by their interaction with the negatively charged SDS molecules. Consequently, all SDS–protein complexes have similar mass-to-charge ratios and electrophoretic mobilities. Future work

will focus on using CGE–SDS, the CE analogue of SDS–PAGE (that has been used to separate model proteins as well as mixtures from complex biological samples). In this separation mode, it is expected that proteins will not only be separated into multiple bands, but also clearly separated from any residual unreacted MTG.

In order to determine if the signal enhancement observed for the mitochondria-enriched fractions corresponds to proteins we used a second protein-selective fluorescent label. For that purpose, an MTG-labeled mitochondria-enriched fraction was labeled with FQ. Upon reaction with primary amines, such as those contained in protein lysine residues [12], this fluorogenic reagent forms a fluorescent isoindole ring (λ_{ex} = ~480 nm, λ_{em} = 590 nm). Fluorescent species containing this moeity were detected at 635 nm (dashed line in Fig. 3). Based on this detection scheme, we reasoned that any proteins labeled with MTG would also be labeled with FQ and that the labeled proteins could be detected simultaneously in separate channels. On the other hand, unreacted MTG will still be detected only at 520 nm. Indeed, shadowing the MTG-labeled species were FQ-labeled species in the 260–280 s migration time range (Fig. 3E, solid and dashed lines, respectively).

In addition to simultaneous detection of MTG-labeled and FQ-labeled species (Fig. 3E), two unexpected features were noticed in the electropherogram: (i) a slight shift in the maximum migration time for the MTG channel and the FQ channel (Fig. 3D solid and dashed lines, respectively); and (ii) a broad band (t = 220–250 s) appeared upon FQ-labeling, which was absent when MTG alone is used for labeling (Fig. 3D). It is important to point out that detection of this band at 520 nm (Fig. 3E, solid line) does not correspond to MTG's labeling but is caused by the broad spectral emission of the FQ-labeled proteins that results in cross-talking.

A protein that is double-labeled with MTG and FQ would have the same migration time in both channels only if the labeling processes resulted in the modification of exactly the same lysine and cysteine residues by the FQ and MTG labeling reactions, respectively. It is known that multiple labeling of proteins is characterized by a different number of fluorescent moeities positioned at different amino acid residues that ultimately result in changes in the electrophoretic mobility [34,35]. In addition, the band migrating at 274–275 s most likely corresponds to a mixture of proteins. Therefore, it is not surprising that signal maximum at 635 nm (t_m = 271 s; RSD 0.4%; n=5) does not match that at 520 nm (t_m = 274 s; RSD 0.4%; n=5).

The broad band (t = 220–250 s) that appeared upon FQ labeling of the mitochondria-enriched fraction (Fig. 3E, dashed line) indicates that species detected under this profile could correspond to other mitochondrial proteins outside the effective labeling range imposed by MTG's subcellular localization or, less likely, to other species that contain primary amine groups [36–38]. If the band at 220–250 s corresponds to proteins they would be mitochondrial proteins found in other submitochondrial compartments (e.g. outer membrane) or other organelles present in the mitochondria-enriched fraction (e.g. lysosomes). Further evidence suggesting that the 220–250 s corresponds to proteins and not to other species with primary amines comes from time-dependent FQ labeling studies that are presented below. All in all, the fact that MTG labeling was unable to report the presence of these proteins, as noticed by comparing the 220–250 s range in Fig. 3E and D, clearly points to the selectivity of MTG afforded by its preferential accumulation in the mitochondrial matrix and the inner membrane side facing the matrix.

As discussed above and shown in Fig. 3B and D, due to the co-migration of MTG-labeled proteins and unreacted MTG, we relied on the fluorescence enhancement observed when MTG covalently binds to proteins. However, this comparison does not allow us to determine whether unreacted MTG is one of the components of an electropherogram (e.g. Fig. 3C and D). In order to investigate this case, we reacted FQ with a MTG-labeled mitochondria-enriched fraction and monitored the changes in migration time vs. reaction time (Fig. 4). As the labeling time with FQ at 40 °C was increased from 0 to 50 min (Fig. 4A–D), migration time increased and the peaks broadened significantly in both channels. This kind of broadening has been attributed to the multiple-labeling of proteins [34] and cannot result from labeling aminated phospholipids with one or two amino groups per molecule [36–38]. Following the

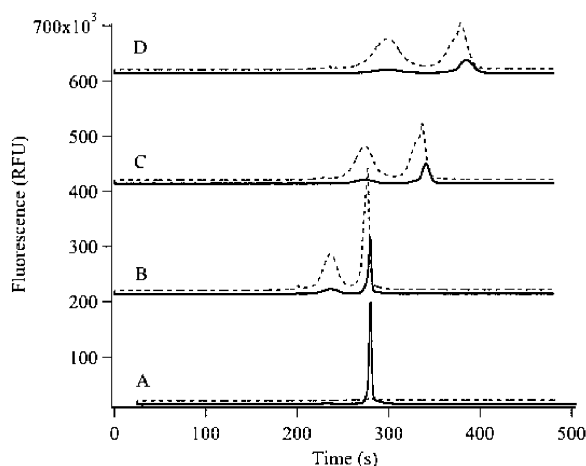

Fig. 4. FQ-labeling reaction of MTG-labeled mitochondrial proteins monitored over time. Samples labeled with FQ for: 0 (A), 15 (B), 30 (C) and 50 min (D). FQ-labeling reaction was carried out at 45 °C. Other conditions as in Fig. 3.

same rationale, the broad band at 220–250 s in Fig. 3E can also be assigned to proteins and not to other species containing one or two primary amine groups per molecule. Also, the addition of FQ labels to a protein changes the protein's net charge and mass and indirectly it may alter protein conformation or its interactions with SDS. The combined effect would be an electrophoretic mobility shift that is indicative of a more negatively charged species. If MTG alone was causing the peak detected in channel 1 (Fig. 4A, solid line) its migration time would remain unchanged upon reaction with FQ. Instead, the whole band shifted upon FQ labeling, ruling out the possibility that the MTG is causing the peak detected in trace A, and confirming that detection at 520 nm truly corresponds to MTG-labeled proteins.

4. Conclusions

MTG, a fluorescent probe that is commonly used in flow cytometry and confocal microscopy for identification and localization of mitochondria, was used as fluorescent protein label. The CE-based strategy for mitochondrial protein analysis presented here exploits the subcellular and chemical selectivities of MitoTracker Green to favor labeling of a subset of proteins found in a mitochondria-enriched fraction. The emergence of protein labels that also have the ability to be directed to specific subcellular compartments promises to be a useful approach for simplifying the analysis of subcellular proteomes.

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