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Technical Note

A Photocross-linking Fluorescent Indicator of Mitochondrial Membrane Potential[1]

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Ionic dyes that distribute across membranes according to electrical potential have proven valuable as fluorescent indicators of mitochondrial energetics in living cells. Applications have been limited, however, as potential-dependent staining is lost during cell fixation. We have produced a membrane potential indicator whose potential-dependent distribution can be made permanent, to enable correlation of membrane potential with cytochemical information from immunofluorescence. A carbocyanine dye was derivatized with a photoreactive nitrophenylazide moiety so that irradiation would induce nonspecific, covalent attachment to nearby molecules. Photo-induced cross-linking was observed in paper chromatography, when irradiation caused immobilization of the dye. The new dye, named PhoCy (photofixable cyanine), showed specific staining of mitochrondria in living Swiss 3T3 fibroblasts. When living cells were stained, irradiated, and fixed with formaldehyde, mitochondrial staining was retained owing to cross-linking with mitochondrial components. Omission of irradiation eliminated mitochondrial staining in fixed cells. Labeling, irradiation, and fixation procedures were optimized to produce bright specific staining with minimal background. The indicator's sensitivity to mitochondrial potential was demonstrated by treating cells with 2,4-dinitrophenol, an uncoupler of mitochondrial electron transport, which decreased mitochondrial staining in living cells and in the corresponding fixed specimens. (*J Histochem Cytochem 41:631–634, 1993*)

KEY WORDS: Mitochondria; Potential; Photoreactive; Cross-linking; Cyanine; Nitrophenylazide; Immunofluorescence; Living cell.

Introduction

Potential-sensitive fluorescent dyes have been valuable for studying membrane potential in living cells and tissues (1–4). Mitochondrial potential has been studied in individual living cells (1–5), using dyes that reflect membrane potential through charge-sensitive partitioning of dye across membranes (5,6). For example, the membrane-permeant cationic probes (e.g., cyanines, safranin, and Janus green) are accumulated to a modest degree in the cytoplasm of hyperpolarized living cells, but they are significantly deposited within mitochondria owing to the large negative mitochondrial membrane potential. The distribution of probe can be visualized and quantified with imaging microscopes and can be monitored in time after perturbation of cells with various pharmacological reagents. These probes enable visualization of potential changes, but unfortunately potential-dependent staining is lost during cell fixation. To investigate relationships between membrane potential and various structural properties that can be visualized with the use of fluorescent antibodies, a method must be developed to "lock in" the potential-dependent staining intensity obtained with the potential sensitive probes. We report here the synthesis and application of a new fluorescent indicator of mitochondrial potential whose potential-dependent staining is retained in fixed specimens.

The indicator was produced by covalently coupling a photoreactive nitrophenylazide moiety (7) to a dye that senses potential through charge-dependent partitioning into mitochondrial membranes. Irradiation of living cells stained with the dye led to covalent cross-linking of the dye to components of the mitochondria. Therefore, after fixation, mitochondrial staining was retained and reflected the potential at the time of irradiation. The indicator preserves, in the fixed sample, a "snapshot" of mitochondrial potential which can be correlated with cell morphology using immunostaining or other techniques made accessible through cell fixation.

Materials and Methods

Organic Synthesis. Dyes have been named using a system of code numbers that encompasses all dyes from ongoing programs in the Waggoner laboratory (8). The dye synthesis is shown in Figure 1. All reagents and materials for synthesis were purchased from Aldrich Chemical (Milwau-

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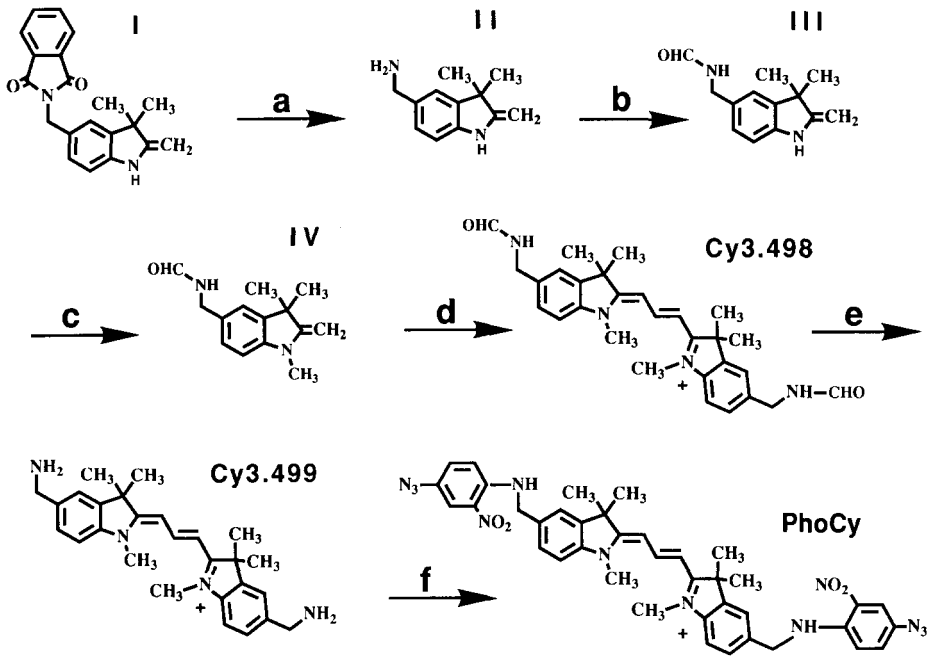

Figure 1. Synthesis of Cy3.500. (a) HCl, CH_3OH; (b) CH_3OCHO; (c) CH_3I; (d) pyridine, $CH(OEt)_3$; (e) HCl, CH_3OH; (f) 4-fluoro-3-nitrophenylazide, NEt_3.

kee, WI). Flash-column chromatography was performed using 60 230–400-mesh silica gel. Silica gel TLC plates were obtained from Analtech (Newark, DE). Absorbance spectra were obtained on a Hewlett–Packard 8452 diode array spectrophotometer, and we are grateful to the mass spectrometry facility of the University of Pittsburgh for providing mass spectral analyses. Proton NMR spectra were recorded on an IBM 300 FT-NMR. Chemical shifts are in parts per million relative to internal tetramethylsilane standard. Abbreviations used in NMR assignments are: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad; Hz, Hertz.

3,3-Dimethyl-2-methylene-5-aminomethylindoline (II). 3,3-dimethyl-2-methylene-5-(phthallimidomethyl)indoline (8) (I) (2 g, 6.3 mmol) (starting materials: 2,3,3-trimethylindolenine, Aldrich catalogue T7,680-5; *N*-(bromomethyl)phthalimide, Aldrich catalog 25,261-1) was dissolved in 50 ml 2:1 concentrated HCl:methanol and refluxed under nitrogen for 19 hr. After cooling to room temperature under nitrogen, water was added and the reaction mixture was extracted with dichloromethane. The aqueous extract was brought to pH 10 with ammonium hydroxide and extracted with ethyl acetate. The ethyl acetate was stirred over sodium sulfate, filtered, and evaporated under reduced pressure to yield 3,3-dimethyl-2-methylene-5-aminomethylindoline as a viscous oil (50% yield): PMR ($CDCl_3$) 7.46, d, 1H (8 Hz); 7.26, s, 1H; 7.20, d, 1H (8 Hz); 3.88, s, 2H; 2.25, s, 3H; 1.28, s, 6H.

3,3-Dimethyl-2-methylene-5-(*N*-formyl)aminomethylindoline (III). 3,3-Dimethyl-2-methylene-5-aminomethylindoline (II) (1.9 g, 10 mmol) was dissolved in 150 ml methyl formate (Aldrich catalogue 17,667-2) in flame-dried glassware and refluxed under nitrogen for 22 hr. Undissolved II went into solution after several hours of reflux. Evaporation of the reaction mixture under reduced pressure produced a nearly quantitative yield of 3,3-dimethyl-2-methylene-5-(*N*-formyl)aminomethylindoline as a viscous oil. PMR ($CDCl_3$) 8.28, s, 1H; 7.48, d, 1H (8 Hz); 7.23, s, 1H; 7.18, d, 1H (8 Hz); 4.52, d, 2H (6 Hz); 2.28, s, 3H; 1.29, s, 6H.

1,3,3-Trimethyl-2-methylene-5-(*N*-formyl)aminomethylindoline (IV). 3,3-Dimethyl-2-methylene-5-(*N*-formyl)aminomethylindoline (III) (2.2 g, 11 mmol) was dissolved in iodomethane (9 g, 63 mmol) (Aldrich catalogue 1-850-7). The solution was heated on a steam bath in a sealed tube for 6 hr and kept sealed at room temperature for an additional 20 hr. The tube was opened and the reaction mixture evaporated to dryness with a steam bath. Residual solid was dissolved in methanol, transferred from the tube, evaporated, triturated with ether, and dried. The resulting solid was 1,3,3-trimethyl-2-methylene-5-(*N*-formyl)aminomethylindoline (>90% yield). PMR (DMSO-d_6) 8.18, s, 1H; 7.86, d, 1H (8 Hz); 7.69, s, 1H; 7.49, d, 1H (8 Hz); 4.42, d, 2H (6 Hz); 3.95, s, 3H; 2.75, s, 3H; 1.51, s, 6H.

Dye Cy3.498. This procedure was based on previously described methods (9). 1,3,3-Trimethyl-2-methylene-5-(*N*-formyl)aminomethylindoline (IV) (1.5 g, 4.4 mmol) was dissolved in 15 ml dry pyridine, and maintained at 100°C under nitrogen. With vigorous stirring, triethylorthoformate (685 mg, 4.6 mmol) (Aldrich catalogue 30,405-0) was added in five portions over 1 hr and the solution was brought to reflux. After 3-hr reflux, additional triethylorthoformate (890 mg, 6 mmol) was added and reflux was continued for 2 hr. Product formation was followed by monitoring the UV absorbance of solution aliquots. The reaction mixture was cooled and ether was added to precipitate the solid red dye Cy3.498, which was collected by vacuum filtration and dried under reduced pressure (>90% yield). PMR (DMSO-d_6) 8.30, t, 1H (14 Hz); 8.16, s, 2H; 7.53, s, 2H; 7.42, d, 2H (8 Hz); 7.36, d, 2H (8 Hz); 6.44, d, 2H (8 Hz); 4.38, d, 4H (14 Hz); 3.65, s, 6H; 1.68, s, 12H.

Dye Cy3.499 (Dihydrochloride Salt). Dye Cy3.498 (1.5 g, 2.6 mmol) was deprotected using the procedure of Dhawan and Southwick (10). It was dissolved in 65 ml 5:60 concentrated HCl:methanol and stirred at room temperature for 12 hr. Absolute ethanol (400 ml) was added, and the solution was evaporated, triturated with ether, and dried to produce solid red dye 3.499 (40% yield). This dye was sometimes recrystallized from isopropanol/ether. PMR (DMSO-d_6) 8.32, t, 1H (14 Hz); 7.82, s, 2H; 7.57, d, 2H (8 Hz); 7.50, d, 2H (8 Hz); 6.50, d, 2H (14 Hz); 4.09, b, 4H; 3.71, s, 6H; 1.70, s, 12H.

PhoCy. Dye Cy3.499 dihydrochloride (107 mg, 0.2 mmol) was dissolved in 20 ml ethanol. Triethylamine (114 mg, 1.1 mmol) (Aldrich catalogue 23,962-3) was added, causing the starting material to dissolve fully. The reaction vessel was covered with foil to exclude light and 4-fluoro-3-nitrophenylazide (600 mg, 3.2 mmol) was added. The solution was stirred

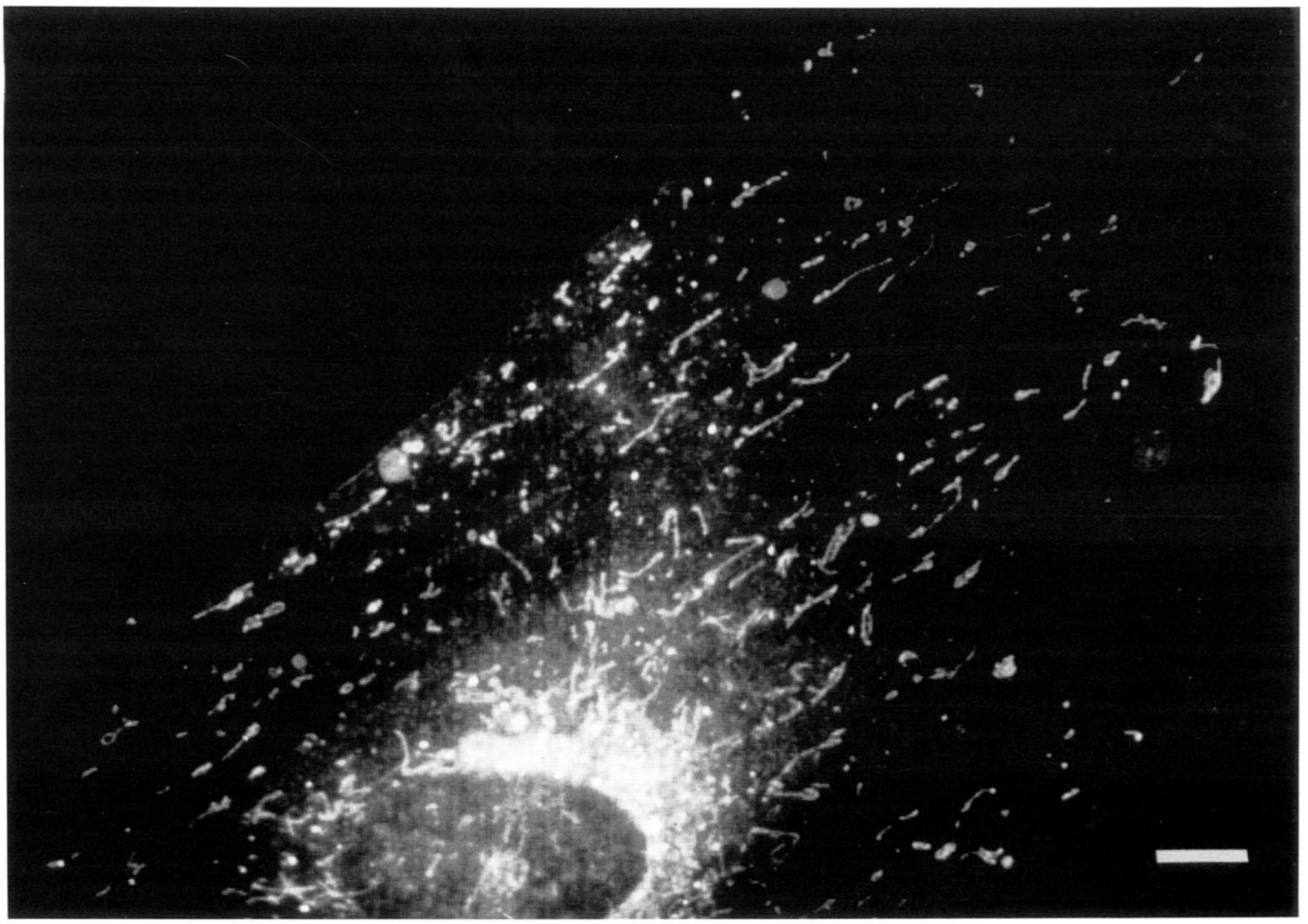

Figure 2. Mitochondrial staining in a Swiss 3T3 fibroblast fixed with formaldehyde. When irradiation was omitted and images were processed identically to the one shown here, no fluorescence was visible. Bar = 5 μm.

under nitrogen at 40°C for 55 hr, then cooled and eluted through a plug of silica gel with 9:1 dichloromethane:methanol to remove polar impurities, which remained adsorbed to the silica. The eluant was evaporated to yield solid red PhoCy, which was dried under reduced pressure. All manipulations were performed at the lowest possible light level. The dye was sometimes purified using silica flash chromatography, eluting with 97:3 dichloromethane:methanol. The reaction and column could be monitored with silica thin-layer chromatography, using 9:1 dichloromethane:methanol. PMR ($CDCl_3$) 8.4, complex overlapping multiplets, 5H; 7.0–7.5, complex overlapping multiplets, 8H; 6.85, d, 2H (14 Hz); 4.62, d, 4H (7 Hz); 3.81, s, 6H; 1.71, s, 12H; FAB MS molecular ion = 739; absorbance λ_{max} = 556 nm; ε = 130,000 M^{-1}.

Paper Chromatography. PhoCy was spotted on strips of filter paper and eluted with methanol. Irradiation was performed for 3 and 8 min using a 6-W hand-held UV lamp (Cole Parmer 9815 Series; mercury vapor 254 nm) held within 2 cm of the paper.

Cell Culture and Preparation. Buffers and other chemicals were obtained from Sigma (St Louis, MO) unless otherwise noted. Swiss 3T3 murine fibroblast cells (CCL92) (American Type Culture Collection; Rockville, MD) were maintained in DME (Dulbecco's modified Eagle's medium), (Gibco; Grand Island, NY), pH 7.4, containing 2 mg/ml penicillin, 0.05 mg/ml streptomycin, and 10% calf serum. Cells were subcultured every 3–4 days by trypsinization using 0.05% trypsin and 0.02% EDTA in a Ca- and Mg-free saline solution (Gibco). All cell cultures were incubated in a humidified 5% CO_2 environment at 37°C. Cell passage number ranged from 122–126.

To obtain well-spread single cells, 5×10^4 cells were plated onto 22-mm^2 coverslips in 60-mm culture dishes in DME containing 0.2% calf serum (serum-deprivation medium). Cultures were typically serum-deprived for 2 days and then processed for mitochondrial staining.

Cell Staining and Imaging. Serum-deprived 3T3 cells were incubated in 100 nM PhoCy in Hanks' balanced salt solution (Gibco) for 14 min at 37°C, rinsed briefly three times in dye-free serum-deprivation medium, and then irradiated for 2 min in this dye-free medium (depth of medium 2 mm). For treatment with 2,4-dinitrophenol, cells were incubated in Hanks' balanced salt solution containing 3 mM 2,4-dinitrophenol and 100 nM PhoCy for 8 min after incubation with dye alone. A 200-W mercury arc lamp (HBO 200) was used, held 50 cm from a mirror directly over the cells. The mirror reflected light into the culture dishes, whose lids were removed during irradiation. After irradiation, cells were fixed as described previously (11) in 2% formaldehyde in Hanks' cytoskeletal stabilizing buffer for 10 min (pH 6.1, 37°C), rinsed three times (5 min each), and mounted in Gelvatol mount-

ing medium (Monsanto; Springfield, MA) containing 30% glycerol with 1% *N*-propyl gallate added as an anti-bleaching agent. Images were obtained at × 100 magnification with a fluorescence microscope equipped with a cooled CCD camera. Fluorescence excitation and emission were carried out through 30-nm bandpass filters with transmission maxima at 530 nm and 580 nm, respectively. All images were background subtracted and histogram stretched (12), and those used for Figure 2 were sharpened (12) before photography of the video image.

Results

The new indicator was made by combining the well-characterized photoreactive nitrophenylazide moiety (7) with a carbocyanine fluorophore that has been successfully applied in many studies as an indicator of mitochondrial potential (1–3,5,6). The influence of hydrophobic side chains on the selectivity and potential sensitivity of this class of indicators has been previously studied (6). This work led us to construct the dye with methyl side chains rather than longer alkyl groups to offset the addition of non-polar nitrophenylazide groups. Protection and deprotection steps increased the length of the synthesis shown in Figure 1, but it was superior in yield to a shorter route in which the phthallimide compound I was simply quaternized, converted to dye, and deprotected to produce Cy3.499. The cross-linking capability of the indicator was demonstrated with paper chromatography. When eluted in the dark, the indicator moved with the solvent front in methanol, but irradiation immobilized it at the origin.

Incubation of Swiss 3T3 fibroblasts in medium containing the indicator produced specific staining of mitochondria with minimal fluorescent background. Preservation of this staining pattern after fixation required careful optimization of irradiation times. Brief irradiation led to fixed specimens in which faint nonspecific staining was the predominant feature. With excess irradiation, fluorescence of the sample was diminished owing to bleaching of the dye.

Formaldehyde fixation was superior to fixation with methanol, acetone, or glutaraldehyde in preserving both mitochondrial fluorescence and cell morphology. Permeabilization with Triton X-100 before fixation decreased mitochondrial staining, as expected.

The sensitivity of cyanines such as PhoCy to membrane potential is well established (6). We tested this property in the new indicator by labeling living cells with medium containing both indicator and 2,4-dinitrophenol, an uncoupler of electron transport (13). The decoupler reduced mitochondrial staining in living cells and led to a parallel reduction in the staining of fixed cells. Qualitative observations indicated that the effect was dose dependent.

Discussion

The new indicator of mitochondrial potential, PhoCy, can be readily synthesized using common methods and simple apparatus. It combines the properties of a well-characterized family of potential sensitive dyes (1–3,5,6) and a photoreactive moiety often used in previous applications, especially photoaffinity labeling (7). The indicator retains the practical advantages of its components, including bright and photostable fluorescence readily visualized in living cells, and efficient photocross-linking with broad specificity (7).

The new indicator enables selective fixation of potential-dependent staining present at a given time in a living cell. We believe it should prove particularly useful in correlating potential with cell morphology studied with immunostaining.

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Literature Cited

1. Waggoner AS. Dye indicators of membrane potential. Annu Rev Biophys Bioeng 1979;8:47
2. Loew LM, ed. Spectroscopic membrane probes. Boca Raton, FL: CRC Press, 1988
3. Waggoner AS. Dye probes of cell, organelle, and vesicle membrane potential. In Martonosi A, ed. The enzymes of biological membranes. Vol. 1. 2nd ed. New York: Plenum Press, 1985:313
4. Freedman JC, Laris PC. Electrophysiology of cells and organelles: studies with optical potentiometric indicators. Int Rev Cytol 1981;12:177
5. Ehrenberg B, Montana V, Wei M, Wuskell JP, Loew LM. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. Biophys J 1988;53:785
6. Sims PJ, Waggoner AS, Wang C, Hoffman JF. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphoatidylcholine vesicles. Biochemistry 1974;13:3315
7. Bayley H. Photogenerated reagents in biochemistry and molecular biology. Amsterdam: Elsevier, 1983
8. Ernst LA, Gupta RK, Mujumdar RB, Waggoner AS. Cyanine dye labeling reagents for sulfhydryl groups. Cytometry 1989;10:3
9. Hamer FM, The cyanine dyes and related compounds. New York: Wiley, 1964
10. Dhawan B, Southwick PL. A convenient preparation of monoaroylpiperazine hydrochlorides. Organic Prepns Proc Int 1975;7:85
11. Conrad PA, Nederlof MA, Herman IM, Taylor DL. Correlated distribution of actin, myosin, and microtubules at the leading edge of migrating Swiss 3T3 fibroblasts. Cell Motil Cytoskel 1989;14:527
12. Castleman KR. Digital image processing. New York: Prentice Hall, 1989
13. Heytler PG. Uncouplers of oxidative phosphorylation. Methods Enzymol 1979;55F:462