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Accelerated Publications

Cell-Permeable Fluorescent Indicator for Cytosolic Chloride†

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ABSTRACT: A major limitation of quinolinium-based fluorescent indicators for cytosolic Cl^- has been the necessity of invasive cell loading because the positively charged ring nitrogen confers high polarity and membrane impermeability. A novel approach to mask the positive nitrogen was developed and evaluated for rapid, noninvasive indicator loading into living cells and effective intracellular trapping. The nonpolar and lipophilic compound 6-methoxy-*N*-ethyl-1,2-dihydroquinoline (diH-MEQ) was Cl^- insensitive but was readily oxidized to the membrane-impermeable and Cl^--sensitive fluorescent indicator 6-methoxy-N-ethylquinolinium chloride (MEQ). MEQ had 344-nm absorbance and 440-nm emission maxima, 0.70 quantum yield, and 4100 M^{-1} cm^{-1} molar extinction coefficient. In aqueous buffers, the fluorescence of MEQ was quenched by Cl^- by a collisional mechanism with a Stern–Volmer constant (K_{Cl}) of 145 M^{-1}. MEQ fluorescence was quenched by other anions (K_{Br} = 275 M^{-1}, K_I = 360 M^{-1}, K_{SCN} = 300 M^{-1}) but not by NO_3^-, SO_4^{2-}, cations, and pH. Swiss 3T3 fibroblasts and colonic T84 cells were loaded with MEQ by incubation at 37 °C with 25–50 μM diH-MEQ for 5–10 min followed by diH-MEQ-free buffer for 15 min. MEQ stained cells brightly and uniformly and was nontoxic in studies of cell growth, cAMP and Ca^{2+} signaling, and electrophysiological properties. MEQ leaked out of cells by <10% in 60 min and was sensitive to cytosolic Cl^- with K_{Cl} = 19 M^{-1}. DiH-MEQ and similar reduced N-substituted dihydroquinolines should have applications in measurements of Cl^- transport and regulatory mechanisms in living cells and epithelia.

Chloride is a major ionic constituent of cells that is involved directly in regulation of cell volume and pH, and secretion of salt and water. Current techniques for measurement of intracellular Cl^- activity include (a) intracellular electrodes, (b) radioactive $^{36}Cl^-$, and (c) Cl^--sensitive fluorescent indicators such as 6-methoxy-*N*-[3-sulfopropyl]quinolinium (SPQ, Figure 1) (Verkman, 1990). The fluorescent indicators were introduced recently (Illsley & Verkman, 1987) and applied initially to the measurement of Cl^- transport in membrane vesicles (Chen et al., 1988; Pearce & Verkman, 1989), liposomes (Verkman et al., 1989b; Garcia-Calvo et al., 1989), and endocytic vesicles (Bae & Verkman, 1990). Cl^- indicators were then used successfully for measurements of Cl^- activity and transport regulation in cells in culture (Chao et al., 1989, 1990; Ram & Kirk, 1989) and in intact epithelia (Krapf et al., 1988a; Shi et al., 1991). In cells, SPQ was found to be nontoxic, uniformly distributed in the cytosolic compartment, sensitive to Cl^- activity, and relatively insensitive to the activities of other cellular ionic constituents.

The major limitation for cellular applications of Cl^--sensitive fluorescent indicators has been their low membrane permeability because of the fixed, positively charged nitrogen on the quinoline ring (see SPQ structure, Figure 1). In organic synthesis studies of structure–function relationships of Cl^--sensitive indicators, it was found that the positive quinolinium nitrogen was essential for Cl^- sensitivity (Krapf et al., 1988b; Verkman et al., 1989a). To overcome the limitation of low cell membrane permeability, a number of invasive loading techniques have been used, including microinjection, hypotonic shock, and scrape loading (Chao et al., 1989; Ram & Kirk, 1989). These techniques introduce uncertain artifacts and are

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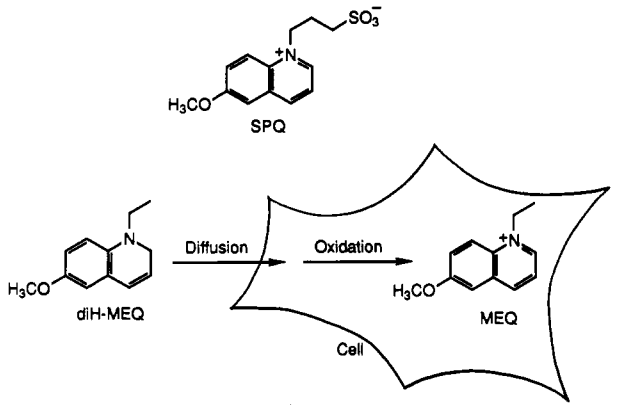

FIGURE 1: Chemical structures of the compounds SPQ, diH-MEQ, and MEQ. DiH-MEQ diffuses across the cell plasma membrane and becomes oxidized in the cell to the membrane-impermeable compound MEQ.

difficult to apply in small cells and cells in intact tissues.

The requirement of a positively charged quaternary nitrogen makes impossible the use of classical "masking" techniques such as addition of acetoxymethyl esters to mask negatively charged carboxyl functions (Minta et al., 1989; Minta & Tsien, 1989; Thomas, 1986). Ester masking confers reasonably high permeability to a number of calcium-sensitive (e.g., fura-2, fluo-3) and pH-sensitive (e.g., BCECF) fluorescent indicators; upon entry into cells, cytosolic esterases cleave the acetoxymethyl ester functions to yield carboxyl functions. The charged indicator is relatively membrane impermeable and thus entrapped in the cytosolic compartment.

We report here the synthesis of a novel Cl^--sensitive fluorescent indicator that permeates into cells rapidly by passive diffusion and remains entrapped in cell cytosol. The compound 6-methoxy-*N*-ethyl-1,2-dihydroquinoline (diH-MEQ, Figure 1) is nonpolar and enters cells easily. In the cytosolic environment, diH-MEQ is oxidized to the polar and membrane-impermeable compound 6-methoxy-*N*-ethylquinolinium chloride (MEQ). MEQ is fluorescent, Cl^--sensitive and selective, and nontoxic in the cellular environment. The synthesis and properties of MEQ and diH-MEQ are reported, and the biological utility is demonstrated in cultured fibroblasts and epithelial cells.

Experimental Procedures

Organic Syntheses. 6-Methoxy-*N*-ethylquinolinium iodide (MEQ) was prepared by reaction of 6-methoxyquinoline (3.2 g, 20 mmol) with iodoethane (6.4 g, 40 mmol) for 1 h under reflux. The precipitate was washed with ether and crystallized from ethanol/water (20:1). 6-Methoxy-*N*-ethylquinolinium iodide was a bright yellow crystalline solid, yield 97%; mp 182–183 °C (decompn); NMR (D_2O, 300 MHz) 7.4–9.1 (m, 6 H), 4.9 (q, 2 H), 3.95 (s, 3 H), 1.6 (t, 3 H); MS single peak, M = 188.

6-Methoxy-*N*-ethyl-1,2-dihydroquinoline (diH-MEQ) was prepared by slow addition of $NaBH_4$ (228 mg, 6 mmol, 12% in H_2O) to 20 mL of an aqueous solution of MEQ (945 mg, 3 mmol) at room temperature under flowing N_2 in the dark, on the basis of the general procedures described by Carroll et al. (1976). The solution turned dark red immediately and then yellow. After 1 h of reaction time, the separated yellow oil was extracted with ether. The solution was dried (Na_2SO_4) and stored at −70 °C in the dark under N_2. To prepare a cell loading solution, the ether was evaporated under an N_2 stream and the yellow oil was dissolved in a physiological buffer. Yield 90%; NMR (DMSO-d_6, 300 MHz) 6.4–6.6 (m, 3 H), 6.24 (d, 1 H), 5.77 (m, 1 H), 3.92 (m, 2 H), 3.63 (s, 3 H), 3.13 (q, 2 H), 1.03 (t, 3 H); MS single peak, M = 189.

Spectroscopic Measurements. Absorbance spectra were measured on a Hewlett-Packard photodiode array spectrometer (Model 8452A). Molar extinction coefficients (M^{-1} cm^{-1}) were measured in 10 × 10 mm cuvettes. Fluorescence measurements were carried out on an SLM 48000 multiharmonic fluorometer. Corrected excitation and emission spectra were measured at 4-nm bandwidths. Quantum yields were calculated from integrated emission spectra by using quinine sulfate in 0.1 N H_2SO_4 as a standard (quantum yield 0.70). Fluorescence quenching titrations were carried out at peak excitation and emission wavelengths. Microliter aliquots of the sodium salt of the quenching anion (1 M) were added to 2 mL of indicator (10 μM) in 5 mM sodium phosphate, pH 7.4. Fluorescence intensities in the absence (F_0) and presence (F) of the anion quencher were measured as a function of anion concentration ([Q]) to give the Stern–Volmer constant (K_q) according to the relation $F_0/F = 1 + K_q[Q]$.

Cell Studies. Swiss 3T3 fibroblasts (ATCC CCL 92, passages 121–128) were grown at 37 °C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum. T84 cells were grown in a 1:1 mixture of Ham's F-12 and DMEM at 37 °C. Cells were grown on 18-mm diameter round glass cover slips for insertion into a continuous perfusion chamber (Chao et al., 1989). Cells were loaded with MEQ by a 5–10-min incubation with 25–50 μM diH-MEQ in a physiological buffer, followed by a 15-min incubation at 37 °C to facilitate uniform intracellular distribution (see Results). After cell loading, fluorescence was monitored in an epifluorescence microscope (Leitz, with Technical Instruments, Inc. coaxial–confocal attachment) using a 40× objective (Leitz, quartz, glycerol immersion, numerical aperture 0.65). Fluorescence was detected by a photomultiplier or microchannel plate intensifier/charged coupled device camera. Calcium signaling studies were performed by addition of 10 nM vasopressin to fibroblasts loaded with fluo-3-AM. Baseline and CPT-cAMP stimulated short-circuit current was measured in T84 cells grown on a porous Nucleopore filter and mounted in an Ussing chamber (Chao et al., 1990).

Results

Figure 1 shows the rationale for design of the cell-permeable Cl^- indicator diH-MEQ. DiH-MEQ is a nonpolar compound (octanol/water partition coefficient 6 × 10^3) that diffuses across lipid membranes rapidly. DiH-MEQ is readily oxidized to MEQ by O_2, oxidized substrates, and intracellular enzymes with oxidative activity (see below). The compound MEQ is fluorescent and more water-soluble than the membrane-impermeable reference Cl^- indicator SPQ (water solubilities: MEQ, 0.5 M; SPQ, 0.12 M) (Krapf et al., 1988b).

The fluorescence spectra of diH-MEQ and MEQ are shown in Figure 2. DiH-MEQ has green fluorescence in an aqueous environment that was insensitive to Cl^-, whereas MEQ has blue fluorescence that was strongly Cl^- sensitive. In the absence of Cl^-, MEQ had a quantum yield of 0.70 and molar extinction coefficients (in M^{-1} cm^{-1}) of 5600 (318 nm), 4100 (344 nm), and 1670 (370 nm). MEQ fluorescence was quenched by Cl^- without change in the shape of the emission spectrum. Stopped-flow measurements showed that MEQ fluorescence responded in <1 ms to changes in Cl^- concentration. Stern–Volmer plots for quenching of MEQ by a series of anions were linear. Stern–Volmer constants (in M^{-1}) were Cl^-, 145; Br^-, 275; I^-, 360; and SCN^-, 300. MEQ fluorescence was not influenced by pH (4–9), NO_3^-, SO_4^{2-}, phosphate, cations, sugars, and urea.

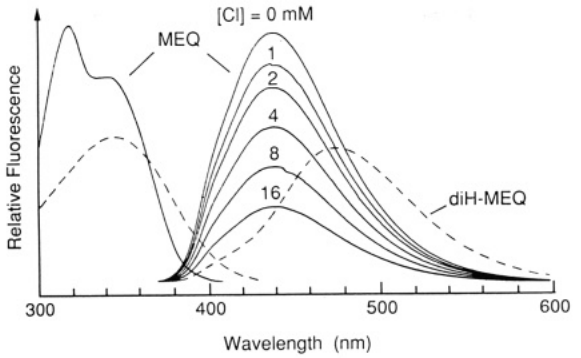

FIGURE 2: Fluorescence properties of diH-MEQ and MEQ. Excitation and emission spectra of diH-MEQ and MEQ in 5 mM sodium phosphate, pH 7.4, containing specified [NaCl].

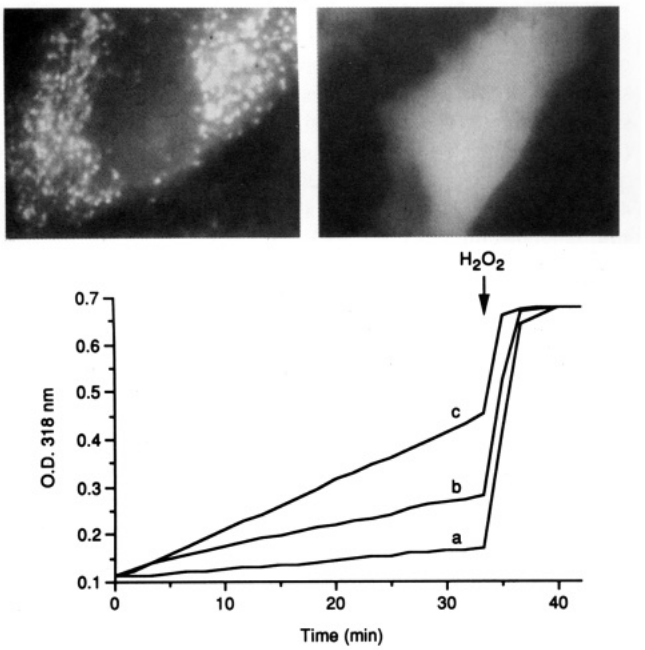

FIGURE 3: Conversion of diH-MEQ to MEQ in cells. (Top) Fluorescence micrographs of Swiss 3T3 fibroblasts recorded at 10 s (left) and 15 min (right) after a 5-min incubation with 50 μM diH-MEQ in Hanks' balanced salt solution (HBSS). The 15-min incubation was carried out in a 37 °C incubator. (Bottom) Time course of conversion of diH-MEQ in 2 mL of a stirred suspension containing 10 μM diH-MEQ in 5 mM sodium phosphate (a) bubbled with N_2, (b) equilibrated with air, and (c) after addition of a muscle cell homogenate (0.05 mg of protein/mL) that was filtered through a 1-μm filter. Where indicated, 3% H_2O_2 was added to oxidize diH-MEQ completely.

Figure 3 (top) shows fluorescence micrographs of Swiss 3T3 fibroblasts incubated with 50 μM diH-MEQ for 5–10 min at 37 °C. Immediately after loading (left), the dye was non-uniformly distributed in vesicular compartments. After a 15-min incubation at 37 °C (right), cells were stained uniformly without significant dye compartmentation. Uniform staining of other cultured cells (T84, MDCK, LLC-PK1, tracheal epithelium in primary culture) and the intact kidney proximal tubule was observed at 15–30 min after staining. Measurement of single-cell fluorescence spectra by an intensified photodiode array spectrometer showed that diH-MEQ was converted completely to MEQ in <10 min.

The kinetics of diH-MEQ conversion to MEQ was studied by absorption spectroscopy. Maximum absorbances of diH-MEQ and MEQ in water were at 344 and 318 nm, respectively. Figure 3 (bottom) shows that diH-MEQ was converted to MEQ at a rate of <0.5% min^{-1} in a deoxygenated cell-free buffer and 0.9% min^{-1} in a buffer equilibrated with room air.

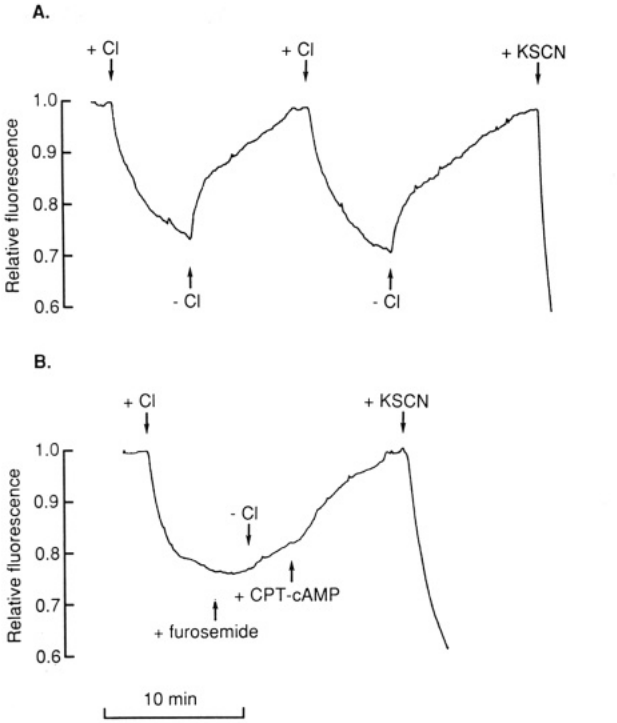

FIGURE 4: Intracellular Cl^- activity measurement by entrapped MEQ. (A) Swiss 3T3 fibroblasts were loaded with diH-MEQ as in Figure 3 (top) and bathed in a physiological saline solution (HBSS) containing 110 mM Na^+, 5 mM K^+, and 100 mM Cl^- or isethionate$^-$. Solution Cl^- content is indicated by arrows. KSCN (150 mM) was added at the end of the experiment to quench intracellular MEQ fluorescence. (B) Stimulation of Cl^- conductance in T84 cells by CPT-cAMP. T84 cells loaded with diH-MEQ were bathed in a solution containing 0 or 100 mM Cl^-. Where indicated, 0.5 mM furosemide and 0.5 mM CPT-cAMP were added.

Addition of a small quantity of cell homogenate increased the conversion rate to 2.0% min^{-1}. These results indicate that cells possess the appropriate substrates and enzymes to facilitate the oxidation of diH-MEQ.

Figure 4A shows the response of MEQ-loaded cells to changes in external Cl^- activity. Intracellular MEQ fluorescence decreased and increased reversibly upon Cl^- addition to and removal from the external solution. In four sets of calibration experiments performed as described previously (Chao et al., 1989), the intracellular Stern–Volmer constant was 19 ± 1 M^{-1}, slightly higher than that reported for SPQ in fibroblasts and other cell types (13–15 M^{-1}) (Krapf et al., 1988a; Chao et al., 1989). A Stern–Volmer constant of 19 M^{-1} corresponds to 50% MEQ quenching at 53 mM Cl^-. The similar fluorescence intensities at 0 Cl^- in Figure 4A indicate a low rate of MEQ leakage. The leakage rate was 8 ± 2% h^{-1} in four experiments with no photobleaching.

Several types of studies were performed to evaluate whether there were toxic effects of cell loading with diH-MEQ. Swiss 3T3 fibroblasts and T84 cells loaded with diH-MEQ and incubated for 72 h at 37 °C with media containing 2 mM MEQ had the same morphology and growth characteristics as unloaded control cells. The signaling pathway for stimulation of Cl^- channel activity by cAMP in T84 cells was intact after loading with diH-MEQ. Figure 4B shows an experiment in which Cl^- was first added to diH-MEQ-loaded T84 cells to increase intracellular Cl^- activity to ~36 mM. The Na-K–2Cl symport inhibitor furosemide was then added to inhibit non-channel-mediated Cl^- efflux. Subsequent removal of solution Cl^- caused slow Cl^- efflux from cells (slow fluorescence increase), which was enhanced markedly by addition of the cell-permeable cAMP analogue chlorophenylthio-cAMP. To

assess K^+ and Cl^- channels electrophysiologically, short-circuit current (I_{sc}) was measured in control and MEQ-loaded T84 cells grown on porous supports. Initial (0.5–0.7 $\mu A\ cm^{-1}$) and CPT-cAMP- (0.5 mM) stimulated (30–40 $\mu A\ cm^{-1}$) I_{sc} values were not affected by indicator loading. In addition, the time course of calcium elevation in Swiss 3T3 fibroblasts loaded with fluo-3-AM was not affected by indicator loading. Finally, the staining of mitochondria by the dye rhodamine 123 (Johnson et al., 1980) was similar in control and MEQ-loaded fibroblasts, suggesting that mitochondrial integrity remained intact. Taken together, these experiments demonstrate that multistep, complex cell functions were not influenced by diH-MEQ loading and oxidation to MEQ.

Discussion

Our goal was to synthesize a compound that could be loaded rapidly into cells by noninvasive means and could undergo chemical conversion to a Cl^--sensitive fluorescent indicator that would remain trapped in the cytosolic compartment. Previous structure–function analyses indicated that Cl^--sensitive indicators with acceptable optical properties and ion selectivities required a quinolinium backbone that was monosubstituted at positions 2–6 with an electron-donating group and *N*-quaternized with an aliphatic side chain (Krapf et al., 1988b; Verkman et al., 1989a; Biwersi et al., 1991). Attempts to synthesize cell-permeable indicators by addition of nonpolar functions containing acetoxymethyl esters were not successful because of the low membrane permeability conferred by the positively charged quinolinium nitrogen.

The data reported here demonstrate that the positively charged nitrogen can be masked by reduction; the positive charge was readily regenerated in the cell by an oxidative mechanism. The idea of reduction of a charged to an uncharged aromatic system to facilitate cellular uptake (Gallop et al., 1984) has been applied to the fluorescent dyes dihydrorhodamine 123 (Emmendorffer et al., 1990) and hydroethidine (Luce et al., 1985; Bucana et al., 1986) and has been proposed as a method to deliver drugs to the brain (Bodor, 1988). Cells contain oxidative enzymes (e.g., $NADP^+$-dependent dehydrogenases) and oxidizing metabolic substrates in lysosomal, peroxisomal, and cytosolic compartments (Barrett, 1984) that facilitate the conversion of diH-MEQ to MEQ. It was found that dissolved O_2 and dilute cell homogenates increased the rate of chemical conversion of diH-MEQ to MEQ. Because diH-MEQ is lipophilic, it is taken up rapidly by passive diffusion across cell plasma membranes and concentrated in intracellular membranes. For cell loading, diH-MEQ can be dissolved directly in aqueous buffers.

The N-substitution of diH-MEQ with an ethyl group conferred low polarity and high membrane permeability. Chemical reduction of SPQ and quinolinium compounds that were N-substituted with the charged moieties 3-(trimethylamino)propyl or acetic acid gave relatively impermeable dihydroquinolines that did not enter cells diffusively. The oxidized compound MEQ was localized to the cytosolic compartment and was membrane impermeable in the fibroblasts and epithelial cells examined. As reported for SPQ (Verkman, 1990), MEQ showed no evidence of cellular toxicity as assessed by effects on cell growth, cAMP and calcium signaling, and electrophysiological properties. MEQ fluorescence was sensitive to and selective for Cl^- with 50% fluorescence quenching at 53 mM Cl^-, an activity well within the physiological range. From measurements of nanosecond lifetimes (Chao et al., 1989), the reduced Cl^- sensitivity of quinolinium indicators in cell cytosol compared to aqueous buffers was due to quenching by fixed intracellular anions. As a general guideline for the application of diH-MEQ/MEQ in new cell types, it is advisable to examine diH-MEQ-loaded cells for evidence of toxicity; when absolute Cl^- activities are required, it is advisable to calibrate MEQ fluorescence vs Cl^- activity by an ionophore (high K^+, nigericin, and tributyltin) or equivalent method (Krapf et al., 1988a).

The spectral properties of MEQ are similar to those of SPQ. MEQ has adequate quantum yield, molar absorbance, and photostability for continuous measurement of intracellular Cl^- activity for >1 h with signal-to-noise ratios exceeding 100 and without photobleaching. MEQ fluorescence is excited within the wavelength range of 320–370 nm by halogen lamp, Hg arc lamp, He–Cd laser (322 nm), or Ar laser (351–367 nm). Because MEQ fluorescence is quenched by Cl^- by a collisional mechanism, the fluorescence response to a change in Cl^- is very rapid (<1 ms) but not accompanied by a change in the shape of the excitation or emission spectrum. Incorporation of a Cl^- insensitive chromophore would be required for measurement of Cl^- activity by an intensity ratio. Preliminary studies indicate that conjugation of a dansyl chromophore (excitation 350–380 nm, emission 490–530 nm) with the quinolinium chromophore (excitation 320–370 nm, emission 430–460 nm) via a spacer chain may provide a dual-emission-wavelength indicator (Biwersi et al., 1991). The dihydroquinoline oxidation approach developed here should be of utility in the design of a cell-permeable dual chromophore indicator that is converted to the impermeable quinolinium chromophore.

The results presented here demonstrate the utility of diH-MEQ as a cell-permeable indicator for cytosolic Cl^-. The low toxicity, good Cl^- sensitivity and selectivity, and relatively bright fluorescence should facilitate studies of Cl^- transport regulation in a variety of cultured cells and cells in intact epithelial tissues. In addition, diH-MEQ has applications in the rapid noninvasive loading of biomembrane vesicles and other structures that have or in which can be incorporated an oxidative mechanism.

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Oxygenase Side Reactions of Acetolactate Synthase and Other Carbanion-Forming Enzymes

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ABSTRACT: Enzymes that mediate carbanion chemistry must protect their reactants from solvent and undesirable electrophiles, such as molecular oxygen. A number of enzymes that utilize carbanionic intermediates were surveyed for O_2-consuming side reactions. Several of these enzymes, acetolactate synthase, pyruvate decarboxylase, class II aldolase, and glutamate decarboxylase, catalyze previously undetected oxygen-consuming reactions, while others such as class I aldolase, [(phosphoribosyl)amino]imidazole carboxylase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, and triosephosphate isomerase do not. Prior to this work, only ribulosebisphosphate carboxylase was known to catalyze an oxygenase side reaction. These new examples indicate that while O_2-consuming side reactions are a more general feature of enzyme-mediated carbanion chemistry than has been previously appreciated, they are not necessarily an inevitable consequence of this chemistry. Expression of an oxygenase activity not only depends on the accessibility of the carbanionic intermediate to molecular oxygen but also may depend on the ability of the enzyme to stabilize the initially formed peroxide anion either through protonation with an appropriate enzymic group or through metal coordination.

The oxygen-consuming reaction of ribulosebisphosphate carboxylase is an undesirable feature of the enzyme and accounts for photorespiration with concomitant inhibition of photosynthesis and plant growth by oxygen (Ogren & Bowes, 1971; Hardy et al. 1978). It has been proposed that the oxygenase activity may be an unavoidable reaction with O_2 of the carbanionic intermediate that normally reacts with CO_2 (Lorimer & Andrews, 1973; Andrews & Lorimer, 1978). If the oxygenase reaction is simply a consequence of carbanion chemistry, then any enzymic reaction that involves oxygen-accessible carbanionic reaction intermediates should have an oxygenase activity. Philipp Christen demonstrated that the carbanionic intermediates of several enzymes (class I and class II fructose-1,6-bisphosphate aldolase, 6-phosphogluconate dehydrogenase, aspartate aminotransferase, and pyruvate decarboxylase) were accessible to various oxidants, such as hexacyanoferrate(III) and tetranitromethane (Healy & Christen, 1973). These results demonstrate that there are a number of enzymes with intermediates capable of reacting with external electrophiles that should in principle also be capable of reacting with molecular oxygen. A number of enzymes that catalyze reactions involving carbanionic intermediates were examined for oxygenase activity in order to determine which factors might be important for expression of an oxygenase activity. Those factors that contribute to discrimination between O_2 and CO_2 are particularly important in the case of ribulosebisphosphate carboxylase, as there is currently a considerable effort devoted to elimination of the oxygenase activity of this enzyme by genetic manipulation (Hartman, 1991).

Experimental Procedures

Materials

The 96.8% $^{18}O_2$, 98% $H_2{}^{18}O$, and [2-^{13}C]pyruvate were obtained from Merck, Sharpe and Dohme isotopes. Acetolactate synthase isozyme II (ALS II)[1] from *Salmonella typhimurium* was prepared as previously described (Schloss & Aulabaugh, 1990). The flavin analogue 5-deaza-FAD was a gift from Professor Colin Thorpe of the University of Delaware. [(Phosphoribosyl)amino]imidazole carboxylase from *Escherichia coli* and the substrate [(phosphoribosyl)-amino]imidazole were a gift from Professor JoAnn Stubbe and

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[1] Abbreviations: TPP, thiamin pyrophosphate; FAD, flavin adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methyl glycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid, ALS II, acetolactate synthase isozyme II; 3-PGA, 3-phosphoglycerate.

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