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Biochemical and Biophysical Research Communications 331 (2005) 351–356
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A general fluorescence-based coupled assay for *S*-adenosylmethionine-dependent methyltransferases

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Received 14 February 2005
Available online 2 April 2005

Abstract

We have developed a simple and sensitive fluorescence-based two-step coupled enzyme assay to report the activity of *S*-adenosylmethionine-dependent methyltransferases. This assay relies on a fluorescein–cystamine–methyl red (FL-S-S-MR) reporter molecule that can be activated by thiols. In the absence of thiols, fluorescence from the reporter is quenched through fluorescence resonance energy transfer between the two chromophores. In this report, we use catechol-*O*-methyltransferase with the addition of *S*-adenosylhomocysteine hydrolase to produce the thiol homocysteine. The presence of homocysteine leads to disulfide bond cleavage in the cystamine tether and fluorescence dequenching as the uncoupled chromophores are diluted into the surrounding media. The sensitivity and specificity of FL-S-S-MR to thiols enabled detection of $\leqslant 1$ μM concentrations of homocysteine, suggesting that this assay is sensitive enough to detect biologically relevant amounts of homocysteine. We believe that this fluorescence reporter approach may be generalizable to all enzymatic or chemical assays that produce thiols.

Keywords: Fluorescence; Homocysteine; Methyltransferases; SAH hydrolase; *S*-Adenosylmethionine; *S*-Adenosylhomocysteine; Catechol-*O*-methyltransferase

Methyltransferases (MTase) are enzymes that are dependent upon *S*-adenosylmethionine (SAM) as a cofactor for activity and are essential for numerous cellular functions including DNA and protein methylation, epinephrine synthesis, and phosphatidylcholine synthesis. The enzymes involved in these reactions use SAM as the methyl donor in the transfer of the methyl group to the target molecule. The reaction is coupled to the concomitant production of *S*-adenosylhomocysteine (SAH), which is then rapidly hydrolyzed in cells by the enzyme *S*-adenosylhomocysteine hydrolase (SAH hydrolase) to adenosine and homocysteine (Hcy). Several methyltransferases, including the enzyme that modifies oncogenic Ras proteins, have recently been recognized as important chemotherapeutic targets [1,2]. Therefore, it has become increasingly important to develop simple, inexpensive, rapid, and sensitive assays for these enzymes. Most existing assays for MTases rely on costly radio-labeled materials, time consuming separation schemes or insensitive absorption measurements to identify the methylated product [3–7]. Recently, a three-step coupled enzymatic assay for salicylic acid carboxyl MTase based on absorption measurements of the thiol-activatable Ellman's reagent that is capable of detecting $\geqslant 4$ μM Hcy has been reported [8].

In this study, we developed a simpler and more sensitive fluorescence-based two-step coupled enzyme assay to report the activity of MTases (Fig. 1). To this end, we describe the synthesis and performance of a fluorescence resonance energy transfer (FRET)-based fluorescein–cystamine–methyl red (FL-S-S-MR) reporter molecule that is activated by Hcy. Fluorescent probes utilizing the FRET principle have been constructed

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doi:10.1016/j.bbrc.2005.03.170

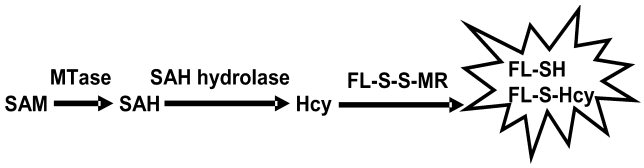

Fig. 1. Detection of homocysteine (Hcy) produced via the enzyme catalyzed methyl transfer and SAH hydrolysis reactions using a thiol-activated FRET-based fluorescent reporter molecule (FL-S-S-MR).

successfully for detecting the existence of genes, proteins, and tumors in vivo because of their high signal-to-background ratio and controllable target specificity [9–11]. In our work, the Hcy generated by the enzymatic reactions can cleave the disulfide bond between the FRET pair and produce a fluorescent signal as the dissociated chromophores diffuse away from each other in the reaction mixture (Fig. 1). The sensitivity and specificity of FL-S-S-MR to thiols enabled us to detect the production of $\leqslant 1$ μM concentrations of homocysteine and allowed for the development of a sensitive coupled assay using porcine catechol-*O*-methyltransferase (COMT) as the model methyltransferase.

Materials and methods

General

^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 300 MHz spectrometer using TMS or solvent peaks as internal reference. Mass spectrometry analysis was performed on either a Hewlett–Packard Engine (EI) or a Finnigan MAT LCQ (ESI) mass spectrometer. Fluorescence measurements were carried out using either a Hitachi F-2000 or Jovin Yvon Fluorolog-3 fluorescence spectrophotometer. A HP 8453 UV/Vis spectrophotometer was used for UV/Vis measurement. All solvents were of analytical grade; the following compounds were distilled under argon before use: tetrahydrofuran (THF) from benzophenone ketyl, dichloromethane (CH_2Cl_2) from P_2O_5. Catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) from porcine liver (lyophilized powder, 1 kU, protein ≈50% by biuret), SAH hydrolase from rabbit erythrocytes (1 U in 0.075 mL of 25 mM Tris, pH 7.4, buffer), 3,4-dihydroxybenzoic acid (DHB), and *S*-(5′-adenosyl)-L-methionine *p*-toluenesulfonate salt (≈90%) were purchased from Sigma. COMT and SAH hydrolase were treated to remove dithiothreitol (DTT) before use (see below). Unless stated otherwise, all other materials were purchased from commercial sources and used as received.

Synthesis of FL-S-S-MR

The fluorescein–cystamine–methyl red (FL-S-S-MR) reporter molecule was synthesized as shown in Fig. 2.

Compound ***1.*** Following a literature method [12], a methanolic solution (100 mL) of cystamine bishydrochloride (2 g, 8.8 mmol), triethylamine (4 mL, 3.3 equiv), and di-*tert*-butyldicarbonate (di-*t*-Boc, 1.96 g, 8.8 mmol) was stirred at room temperature for 20 min. After evaporation of the solvent, the white residue was treated with 1 M NaH_2PO_4 (40 mL, pH 4.2) and extracted with ether (25 mL 2×) to remove the di-*t*-Boc-cystamine. The aqueous solution was basified with 1 N NaOH to pH 9.0, and extracted with EtOAc (20 mL 5×). The organic phase was combined and dried over $MgSO_4$, filtered, and evaporated to yield a slightly yellowish oil-like product (0.88 g, 40%). ^{1}H NMR ($CDCl_3$) δ: 1.44 (s, 9H, *t*-BuO), 1.59 (s, 2H, NH_2), 2.79 (m, 4H, CH_2S), 3.00 (t, 2H, CH_2N, $J = 6.0$ Hz), 3.42 (q, 2H, CH_2NBoc, $J = 6.3$ Hz), 5.68 (bs, 1H, NHCOO) ppm. ^{13}C NMR ($CDCl_3$) δ: 28.4, 38.3, 39.3, 40.4, 42.3, 79.1, 155.8 ppm. MS (EI, *m/z*): 252 (M^+).

Compound ***2.*** Compound **1** (0.49 g, 2 mmol), methyl red (0.59 g, 2 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (DCC, 0.5 g, 2.5 mmol) [13] were dissolved in chloroform (15 mL). After stirring at room temperature for 2 h, the solution was filtered to remove *N*,*N*′-dicyclohexylurea and the solvent was evaporated. The residue was purified by alumina column chromatography using chloroform as eluent.

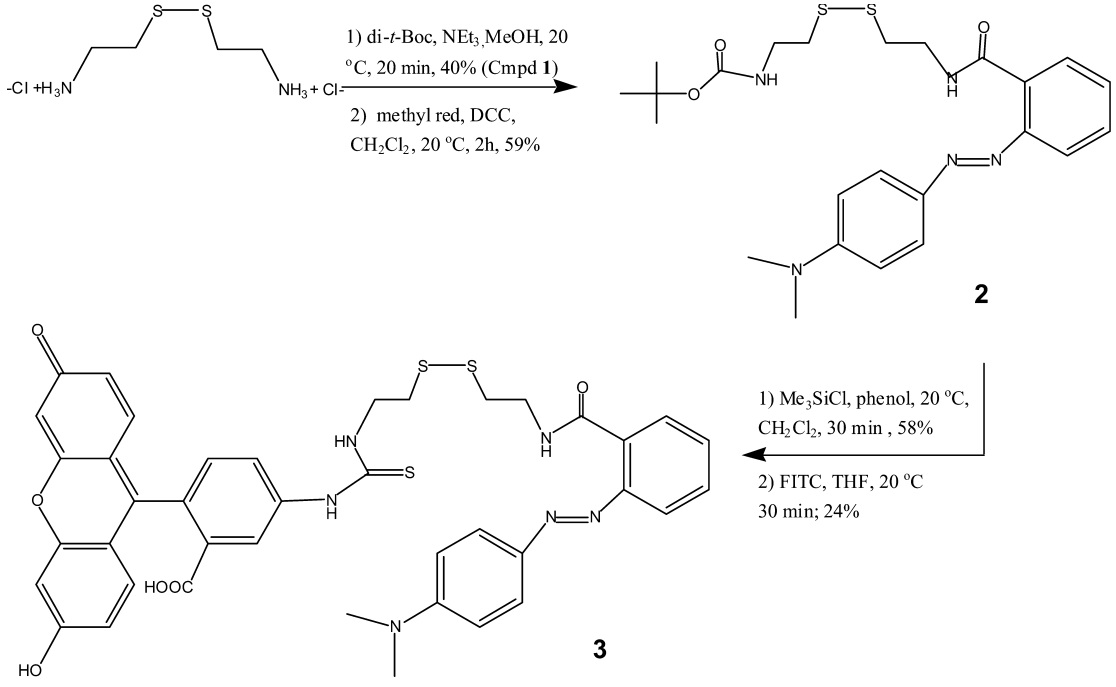

Fig. 2. Synthesis scheme for the thiol-activated fluorescent reporter molecule, fluorescein–cystamine–methyl red (FL-S-S-MR).