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A general fluorescence-based coupled assay for *S*-adenosylmethionine-dependent methyltransferases

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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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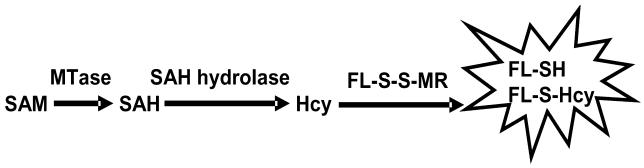

Fig. 1. Detection of homocysteine (Hcy) produced via the enzyme catalyzed methyl transfer and SAH hydrolysis reactions using a thiol-activated FRET-based fluorescent reporter molecule (FL-S-S-MR).

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.

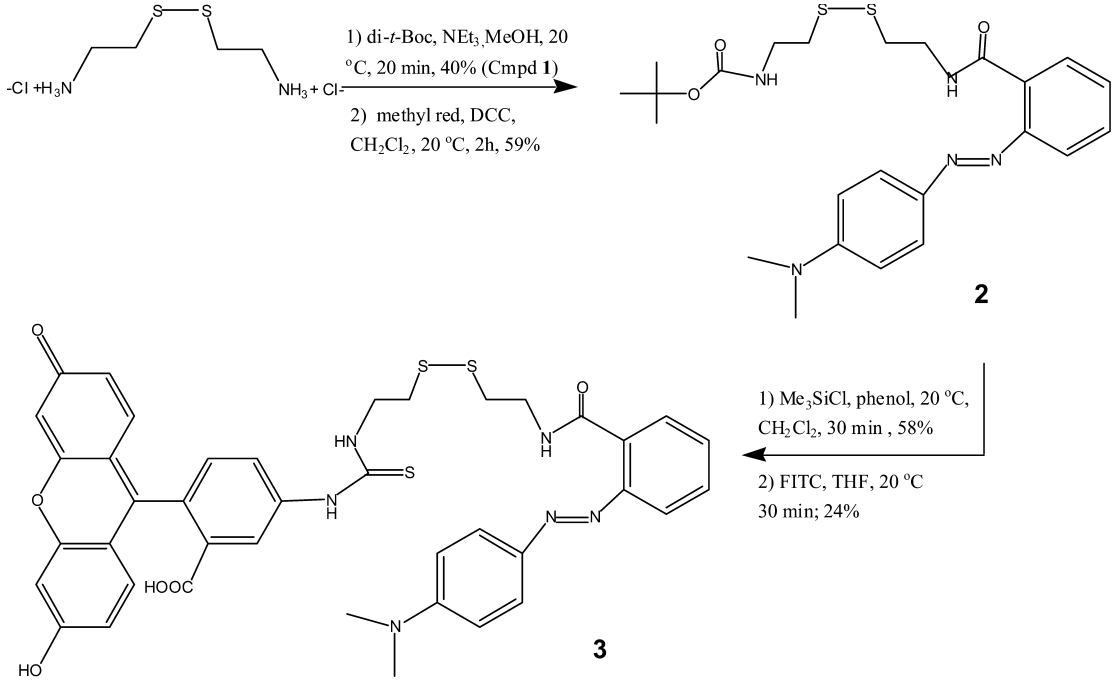

Fig. 2. Synthesis scheme for the thiol-activated fluorescent reporter molecule, fluorescein–cystamine–methyl red (FL-S-S-MR).

Evaporation of the desired fraction gave a red solid product (0.59 g, 59%). ^{1}H NMR ($CDCl_3$) δ: 1.43 (s, 9H, *t*-BuO), 2.79 (t, 2H, CH_2S, $J = 6.3$ Hz), 2.96 (t, 2H, CH_2S, $J = 6.3$ Hz), 3.13 (s, 6H, $(CH_3)_2N$), 3.41 (q, 2H, CH_2NBoc, $J = 6.3$ Hz), 3.87 (q, 2H, CH_2N-methyl red, $J = 6.0$ Hz), 5.18 (bs, 1H, NHCOO), 6.78 (d, 2H, ArH, $J = 9.0$ Hz), 7.49 (quint d, 2H, ArH, $J_1 = 7.4$ Hz, $J_2 = 1.7$ Hz), 7.78 (dd, 1H, ArH, $J_1 = 7.5$ Hz, $J_2 = 1.5$ Hz), 7.83 (d, 2H, ArH, $J = 9.0$ Hz), 8.39 (dd, 1H, ArH, $J_1 = 7.2$ Hz, $J_2 = 1.8$ Hz), 9.52 (t, 1H, NHCO, $J = 5.7$ Hz). ^{13}C NMR ($CDCl_3$) δ: 28.5, 38.0, 38.4, 38.7, 39.5, 40.3, 79.3, 111.7, 116.1, 126.0, 129.1, 129.4, 131.4, 131.8, 143.4, 150.6, 153.2, 156.0, 166.5 ppm. MS (ESI, *m/z*): 504 ($(M + H)^+$), 526 ($(M + Na)^+$).

*Compound **3** (FL-S-S-MR).* To remove the protecting *t*-Boc group in **2**, trimethylchlorosilane (1 M in CH_2Cl_2, 2.5 mL, 20 mmol) and phenol (3.76 g, 40 mmol, dissolved in 10 mL dry CH_2Cl_2) [14] were mixed and added to a CH_2Cl_2 solution (10 mL) of **2** (2.6 g, 5 mmol). After 30 min stirring under argon at room temperature, the solvent was evaporated. The residue was re-dissolved in 100 mL chloroform, washed with 0.5 N NaOH (20 mL 2×) and H_2O (20 mL 2×), and dried over $MgSO_4$. After removal of the solvent, the residue was purified via silica gel column chromatography using a CH_2Cl_2:MeOH step gradient elution (ratio changing from 100:0 to 5:1) to yield a thick red oil (1.17 g, 58%). ^{1}H NMR ($CDCl_3$) δ: 1.57 (s, 2H, NH_2), 2.75 (t, 2H, CH_2N, $J = 6.0$ Hz), 2.95 (t, 4H, CH_2S, $J = 6.0$ Hz), 3.14 (s, 6H, $(CH_3)_2N$), 3.89 (q, 2H, CH_2N-methyl red, $J = 6.0$ Hz), 6.75 (d, 2H, ArH, $J = 9.0$ Hz), 7.47 (m, 2H, ArH), 7.74 (d, 1H, ArH, $J = 7.4$ Hz), 7.81 (d, 2H, ArH, $J = 9.6$ Hz), 8.36 (dd, 1H, ArH, $J_1 = 6.8$ Hz, $J_2 = 2.6$ Hz), 9.54 (t, 1H, NHCO, $J = 5.7$ Hz). TLC and ^{1}H NMR indicated decomposition of this product with time, mainly due to disulfide bond cleavage. Therefore, it was used for the next step immediately after purification.

A THF solution (5 mL) of cystamine–methyl red (0.28 g, 0.69 mmol) was added to a THF solution (5 mL) of fluorescein isothiocyanate (FITC, isomer I, 90% pure, 0.30 g, 0.69 mmol). The mixture was stirred at room temperature under argon for 30 min. After removal of solvent, the residue was dissolved in a small amount of acetone and purified via silica gel chromatography using EtOAc:petroleum ether (80:20) as eluent. The red solid was further purified by re-dissolving it in hot methanol and precipitating it by addition of petroleum ether to yield the dark red solid product (0.13 g, 24% yield). ^{1}H NMR (acetone-d_6) δ: 3.02 (t, 2H, CH_2S, $J = 7.2$ Hz), 3.09 (m, 8H, $(CH_3)_2N$ and CH_2S), 3.88 (q, 2H, CH_2N-methyl red, $J = 6.6$ Hz), 3.97 (q, 2H, CH_2N-fluorescein, $J = 5.7$ Hz), 6.60–6.74 (m, 6H, ArH), 6.86 (d, 2H, ArH, $J = 9.0$ Hz), 7.10 (d, 1H, ArH, $J = 8.1$ Hz), 7.48 (td, 1H, ArH, $J_1 = 7.5$ Hz, $J_2 = 0.9$ Hz), 7.56 (td, 1H, ArH, $J_1 = 7.5$ Hz, $J_2 = 1.5$ Hz), 7.76–7.85 (m, 4H, ArH), 8.02 (t, 1H, NHCS, $J = 3.3$ Hz), 8.12 (dd, 1H, ArH, $J_1 = 7.8$ Hz, $J_2 = 1.5$ Hz), 8.40 (dd, 1H, ArH, $J_1 = 5.7$ Hz, $J_2 = 1.5$ Hz), 8.96 (s, 1H, NHCS), 9.25 (t, 1H, NHCO, $J = 5.7$ Hz), 9.62 (s, 1H, ArH). ^{13}C NMR ($CDCl_3$) δ: 36.7, 37.9, 39.1, 39.5, 42.7, 102.5, 111.0, 111.8, 112.5, 116.2, 117.5, 124.0, 125.9, 127.5, 129.2, 129.3, 129.7, 130.3, 130.8, 131.6, 141.5, 143.3, 148.3, 150.5, 152.6, 153.6, 159.4, 167.3, 168.4, 181.7 ppm. MS (ESI, *m/z*): 791 ($(M - H)^+$), 793 ($(M + H)^+$).

Protocol for FL-S-S-MR fluorescence analysis

A 0.63 mM FL-S-S-MR stock solution in 1 M Tris–HCl, pH 8.0, buffer was stored at −10 °C. This solution was thawed and diluted in buffer to the desired concentrations immediately before use. The maximum excitation and emission peaks of FL-S-S-MR at low concentrations (<20 μM) in Tris buffers are 493 and 516 nm, respectively; these values were used in all measurements.

Calibration for homocysteine detection with FL-S-S-MR

FL-S-S-MR (20 μM) and homocysteine of different concentrations were mixed in 100 mM Tris, pH 7.6, buffer solution and incubated at 30 °C (in air) for 1 h. The fluorescence intensity was then measured at $\lambda_{ex} = 493$ nm and $\lambda_{em} = 516$ nm. The fluorescence difference between each sample and the control, which contained FL-S-S-MR only, was plotted as a function of the homocysteine concentration in the sample. The average of two sets of experimental data is shown in Fig. 6.

Treatment of catechol-O-methyltransferase and SAH hydrolase

To remove DTT from catechol-*O*-methyltransferase (COMT) and SAH hydrolase, 1 mL of 100 mM Tris–HCl, pH 7.9, or 100 mM Tris–HCl, pH 7.5, was added to the two proteins, respectively. Using Amicon Ultra-15 centrifugal filter devices, the solutions were concentrated separately to approximately 250 μL by centrifugation at 4 °C. This procedure was repeated several times until the DTT originally present in the enzymes was diluted approximately 15,000 times. The protein solutions were then divided into small aliquots, stored at −70 °C, and thawed on ice immediately before use.

Protocol for the coupled enzyme assay

In a 150 μL reaction mixture, the final concentrations were 0.72 mM SAM, 0.9 mM DHB, 0.9 mM $MgCl_2$, 0.1 mM EDTA, 0.1%(w:v) BSA, 100 U COMT, and 0.1 U SAH hydrolase in 100 mM Tris, pH 7.6. The reaction mixture was incubated in argon atmosphere at 37 °C first without hydrolase for 15 min, and then with hydrolase for an additional 1 h at 37 °C. Following the incubation, aliquots of FL-S-S-MR solution were added, mixed rapidly, and the fluorescence measurement at 30 °C was initiated immediately. The control experiment was identical except that SAM was replaced with an equal amount of water.

Results and discussion

Design and synthesis of the fluorescent reporter FL-S-S-MR

The FL-S-S-MR molecule was prepared on a 130 mg scale using cystamine as a bi-functional disulfide tether linking the fluorescence donor, fluorescein, to the acceptor, methyl red (Fig. 2) [15]. Fluorescein is extensively used in oligonucleotide molecular beacons [16]. Methyl red is the structural isomer of DABCYL, the universal quencher for chromophores in molecular beacons [17]. We chose methyl red as the acceptor in our reporter molecule as it is readily available, non-fluorescent, and has an absorption spectrum that overlaps with the emission spectrum of fluorescein. The 90° X–S–S–X dihedral angle in disulfide molecules [18] places the fluorescein and methyl red substituents of our construct in close proximity. It is known that FRET takes place when a pair of fluorescence donor and acceptor molecules are separated by 20–100 Å, and contact energy transfer occurs when the two are even closer [17]. In an effort to understand the fluorescence quenching mechanism in our FL-S-S-MR molecule, the distance between the two chromophores was estimated. Based on B3LYP/6-31G* level calculations [19,20] of a simple model compound in which the two chromophores were replaced by two phenyl groups, the moieties are approximately

19.1 Å apart when the molecule is fully stretched. We further compared the UV absorption spectra of the chromophores and found that their combined spectra are similar to that of the FL-S-S-MR molecule (data not shown). Together, these data indicate that FRET is the primary mechanism of quenching [17].

Sensitivity and specificity of the fluorescent reporter FL-S-S-MR to thiols

The ability of the FL-S-S-MR reporter to be activated by thiols was first tested using dithiothreitol (DTT). These experiments established that at concentrations of 1 μM FL-S-S-MR and 100 μM DTT, the fluorescence intensity increased by a factor of 16 after the reaction reached equilibrium (Fig. 3). The excitation and emission spectra of 1 μM FL-S-S-MR reporter before and after the addition of DTT are shown in Fig. 3.

Ideally, our probe should respond specifically to thiols. However, disulfides are susceptible to cleavage by a variety of nucleophiles and some electrophiles, even at room temperature [18]. Since our FL-S-S-MR molecule is designed for enzymatic methyltransferase reactions, we tested it with several other compounds that may be present in typical biochemical assays. The data shown in Fig. 4 demonstrate that the FL-S-S-MR reporter is thiol-specific at room temperature, with activation detectable within 10 min. In addition to DTT, FL-S-S-MR activation was detected in the presence of cysteine and Hcy, but not methionine, SAM, SAH, or 3,4-dihydroxybenzoic acid (DHB), the model COMT methyl-accepting substrate used in our coupled assays.

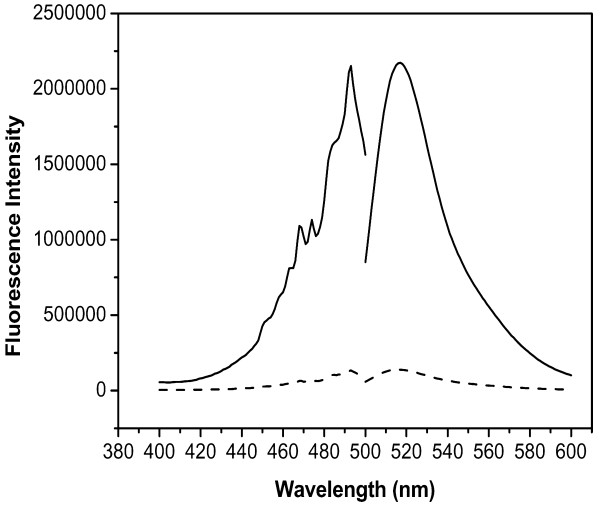

Fig. 3. Excitation (left, $\lambda_{em} = 516$ nm) and emission (right, $\lambda_{ex} = 493$ nm) spectra of FL-S-S-MR before (---) and after (—) activation by DTT. The spectra after the addition of DTT were obtained 100 min after mixing DTT (20 μL, 10 mM DTT solution) with the FL-S-S-MR solution (2 mL of 1 μM FL-S-S-MR in 600 mM Tris–HCl, pH 8.0) at room temperature.

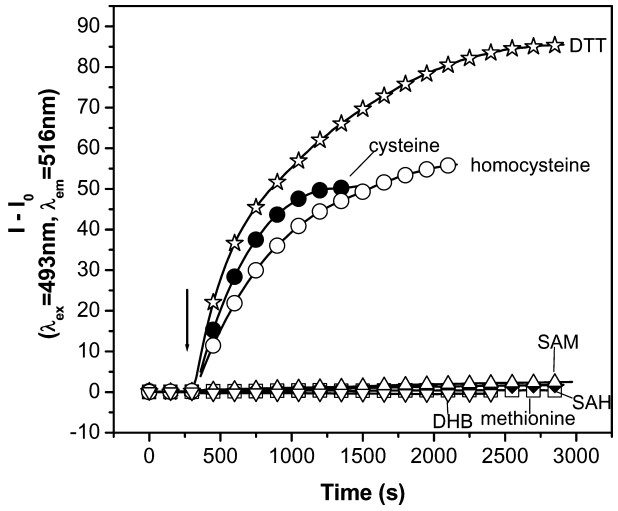

Fig. 4. Fluorescence intensity of FL-S-S-MR reporter in response to different substrates. I_0 is the intensity at time = 0. At time = 300 s (indicated by the arrow), 20 μL of a substrate solution was added to 2 mL of a 1 μM FL-S-S-MR solution in 600 mM Tris, pH 8.0, at room temperature. Substrate concentrations were 500 μM, except SAH which was 100 μM. SAM was dissolved in DMSO; all the other substrates were dissolved in H_2O. No change in fluorescence was observed upon addition of equivalent volumes of DMSO or H_2O to the FL-S-S-MR solution.

Application of FL-S-S-MR in a coupled enzyme assay for COMT, a SAM-dependent MTase

The coupled assay shown schematically in Fig. 1 relies on the presence of SAH hydrolase to convert SAH to Hcy and adenosine. The added benefit of converting SAH to adenosine and Hcy is that any feedback inhibition of the MTase by its product SAH is minimized [8,21]. Therefore, we first examined FL-S-S-MR reporter activation in the presence of SAH hydrolase and SAH, neither of which alone is capable of promoting significant activation of FL-S-S-MR (Figs. 4 and 5). After removal of the DTT in the SAH hydrolase storage buffer, ⩽1 μM concentrations of Hcy either introduced into the reaction mixture or produced in vitro by incubating 1 μM SAH with SAH hydrolase at 30 °C for 1 h were detectable relative to the SAH-deficient control using 20 μM FL-S-S-MR (Fig. 5). These curves approach saturation because activation of the FL-S-S-MR reporter results in the homocysteine molecule being linked to one of the chromophores and thus unavailable for another cleavage reaction. Once all the homocysteine is depleted, no further increase in fluorescence is observed. Compared to a recently published colorimetric MTase assay that was able to detect Hcy generated from 4 μM SAH using 100 μM Ellman's reagent (DTNB) [8], our assay is at least four times more sensitive and uses only one-fifth of the reporter molecule concentration (20 μM FL-S-S-MR). We further generated a standard curve using 20 μM FL-S-S-MR and increasing concen-

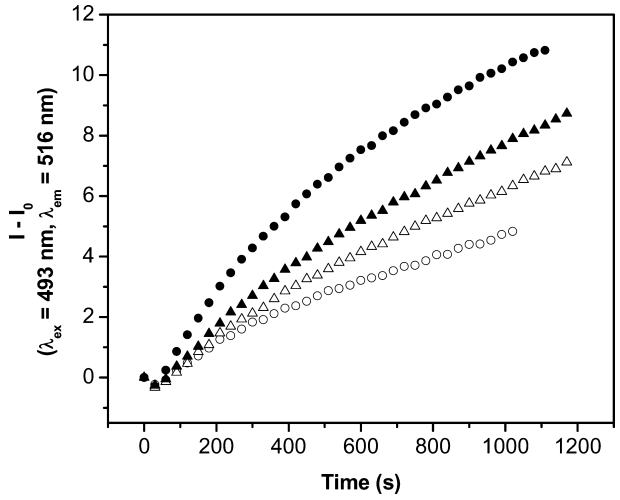

Fig. 5. Detection of homocysteine using the FL-S-S-MR reporter molecule. FL-S-S-MR (20 μM) in 100 mM Tris–HCl, pH 7.5, alone (○), with 1 μM homocysteine (●), with 0.13 U SAH hydrolase alone (△), or with 0.13 U SAH hydrolase + 1 μM SAH (▲) incubated at 30 °C for 1 h prior to the addition of FL-S-S-MR. Fluorescence was measured at 30 °C immediately upon addition of FL-S-S-MR.

trations of Hcy that allowed us to reproducibly correlate the fluorescent signal to the amount of Hcy generated by the reaction (Fig. 6). Additional standard curves can be generated for the desired concentration of FL-S-S-MR used in individual experiments. These data suggest that our fluorescence-based assay has the sensitivity to detect biologically and enzymatically relevant amounts of Hcy under the appropriate assay conditions.

In the coupled enzyme assay, porcine liver COMT and SAH hydrolase (both after DTT removal) were incubated with SAM to catalyze the methyl transfer

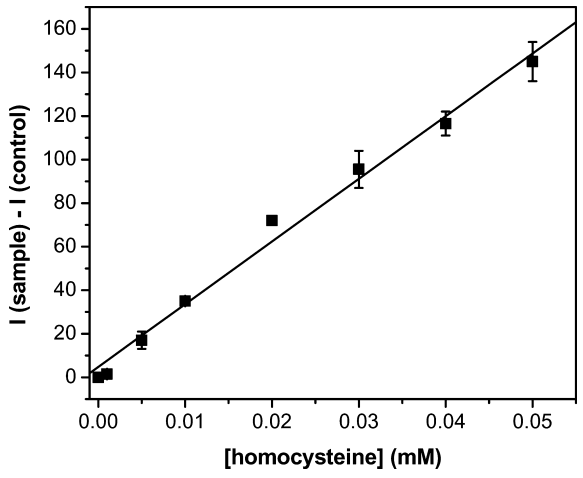

Fig. 6. Standard curve demonstrating the detection of increasing concentrations of homocysteine using FL-S-S-MR (20 μM) in 100 mM Tris–HCl, pH 7.6. The reporter FL-S-S-MR and homocysteine of different concentrations were incubated at 30 °C (in air) for 1 h prior to fluorescence measurement at room temperature. These data represent the average of two experiments and the R^2 value for the linear fit was 99.5%.

and SAH hydrolysis processes. The reaction containing 100 U COMT, 0.9 mM $MgCl_2$, 0.1% BSA, 0.72 mM SAM, and 0.9 mM DHB in 100 mM Tris–HCl was incubated for 15 min at 37 °C, followed by the addition of 0.1 U SAH hydrolase and incubation for an additional hour. Following these incubations, 10 μM FL-S-S-MR was added and detection of fluorescence was started immediately (Fig. 7). These data demonstrate that within 5 min of adding the FL-S-S-MR, the assay generates measurable fluorescence by cleaving and dequenching the reporter molecule. The control reactions not containing SAM showed only modest probe activation under the same conditions (Fig. 7). Since in this type of stopped point assay we added the reporter molecule after the enzymatic generation of Hcy, we first observed rapid onset of fluorescence due to the accumulated Hcy from the reactions, followed by slower signal generation from continued enzyme activity. Together, these data demonstrate that in this single point assay, we can reproducibly measure specific FL-S-S-MR activation by the Hcy end product of the coupled reactions. Furthermore, we were able to detect quantifiable activity above background using only 1 μM FL-S-S-MR (Fig. 7), which is one hundred times less reporter compound than previously reported for the three-step assay using Ellman's reagent [8]. The slight activation of FL-S-S-MR observed in the control experiment not containing SAM is most likely due to residual DTT associated with the enzymes and the subsequent decrease in fluorescence intensity can be attributed to fluorescein photobleaching.

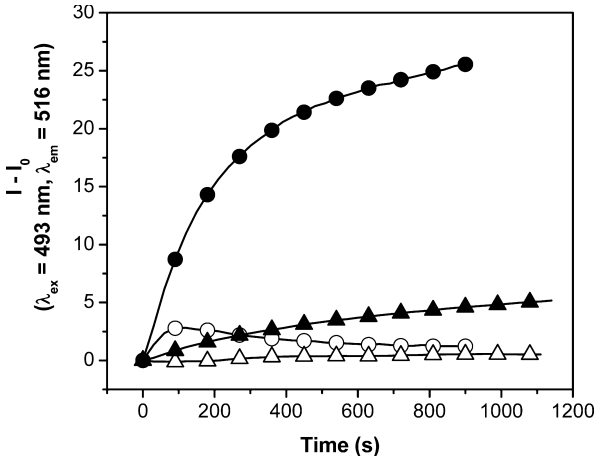

Fig. 7. Coupled enzyme (SAH hydrolase and COMT) assay using FL-S-S-MR as the reporter molecule. COMT (100 U), 0.72 mM SAM, and 0.9 mM DHB, 0.9 mM $MgCl_2$, EDTA, and 0.1%(w:v) BSA in 100 mM Tris, pH 7.6, were incubated in argon atmosphere at 37 °C for 15 min. SAH hydrolase (0.1 U) was added and the incubation was continued for an additional 1 h. At the end of incubation FL-S-S-MR was added and the fluorescence measurement at 30 °C was initiated immediately. The final concentration of FL-S-S-MR in the reaction mixture was either 10 μM (●, ○) or 1 μM (▲, △). The filled symbols represent reactions containing SAM and the empty symbols represent control experiments in which the SAM was excluded.

In conclusion, we have developed a simple and sensitive two-step coupled enzymatic activity assay for SAM-dependent MTases that uses a thiol-activated fluorescent reporter molecule. This methodology provides a viable alternative to radiolabel-based assays and other colorimetric assays that require higher concentrations of reporter molecule and additional enzymatic steps. Although inherently less sensitive than radioassays, our method is faster, less expensive to perform, and can be easily adapted for a high-throughput platform. In addition to the point assay described here, this methodology can also be modified readily for experiments designed to measure enzyme activity in a continuous, real-time manner. We believe that this fluorescence detection approach should be generalizable for all enzymatic or chemical reactions that produce thiols. Work aimed at optimizing the properties of the reporter to improve its detection sensitivity and specificity (e.g., selectivity of Hcy over other thiols) is in progress. Designing this specificity into the reporter molecule will simplify the application of this method to numerous biological systems by eliminating the requirement for removal of thiol-based reducing agents that are commonly used to preserve protein structure and function.

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