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Fluorescent Indicator Displacement Assay for Ligand−RNA Interactions

Jinhua Zhang, Shiori Umemoto, and Kazuhiko Nakatani*

Department of Regulatory Bioorganic Chemistry, The Institute of Scientific and Industrial Research (ISIR), Osaka University, Ibaraki 567-0047, Japan

Received January 6, 2010; E-mail: nakatani@sanken.osaka-u.ac.jp

Since the discovery of a role for RNA in modulating gene expression, functional RNA has become an attractive drug target.[1] Ligand−RNA interactions have been studied using covalent labeling of either the ligand or RNA with fluorescent dyes and by immobilization on sensing devices.[2] However, it is desirable to avoid chemical modification of the ligand or RNA, as this may alter their native secondary and tertiary structures.[3] Displacement assays[4−6] satisfy this criterion because neither covalent labeling nor immobilization is necessary. We describe a fluorescent indicator displacement assay for ligand−RNA interactions based on a novel fluorescent indicator, a 2,7-disubstituted 9*H*-xanthen-9-one derivative (X2S) (Figure 1). X2S is nonfluorescent when bound to RNA but fluoresces when it is displaced from RNA.

The fluorescence of xanthone derivatives is sensitive to the environment in which they are contained.[7,8] The emission of X2S (453 nm, $\Phi_F = 0.789$) in a buffer solution decreased as the concentration of double-stranded RNA (dsRNA) and RNA containing a single-nucleotide bulge increased. The quenching of X2S fluorescence was weakly affected by the bulge nucleotide and decreased in the order A > G > C > U > dsRNA (Figure 2a and Figure S1 in the Supporting Information). Curve fitting analysis suggested 1:1 binding of X2S to the U-bulge RNA with an apparent binding constant of $\sim 1 \times 10^7$ M^{-1}. X2S fluorescence was effectively decreased by a model RNA of the Rev responsible element (RRE) of HIV-1 mRNA consisting of hairpin and stem loops. At concentrations of 1 μM RNA and 2 μM X2S, the fluorescence intensity was 11% for RRE but 39 and 17% for U-bulge RNA and hairpin RNA lacking the stem loop of RRE, respectively (Figures S2 and S3), suggesting that X2S binds to RRE at more than one site. The following spectroscopic and binding properties of X2S make it a useful fluorescent indicator for displacement assays: (1) it binds more efficiently to a region containing unpaired bases than to the double-strand region, and (2) its fluorescence decreases upon binding to RNA. An X2S displacement assay was developed using ligand binding to RRE, one of the most studied RNAs for ligand binding.

Binding of the viral protein Rev to the stem loop of RRE is necessary for nuclear export of the unspliced full-length mRNA of HIV-1 to the cytoplasm and thus is essential for viral replication.[9] Ligands that compete with Rev in binding to RRE have been identified as potential lead compounds for suppressing HIV-1 replication.[10] The fluorescence intensity of a mixture of 2 μM X2S and 2 μM RRE was ~10% of that of X2S alone (Figure 3). The Rev model peptide[11] was added gradually to the preformed X2S−RRE complex, keeping the X2S and RRE concentrations constant. Fluorescence intensity increased as the concentration of the Rev model peptide increased, reaching 80% of that of free X2S at about 2 molar equiv of the peptide. Titration of the preformed X2S−RRE complex with neomycin B, an aminoglycoside antibiotic that binds to RRE in a less specific manner than Rev,[10] resulted in a steady increase in fluorescence (Figure 3b), which reached

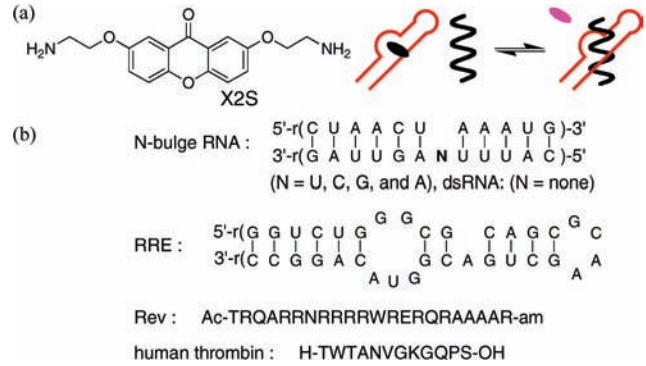

Figure 1. (a) Structure of X2S and illustration of the fluorescent indicator displacement assay. (b) RNA nucleotide and peptide amino acid sequences used in these studies.

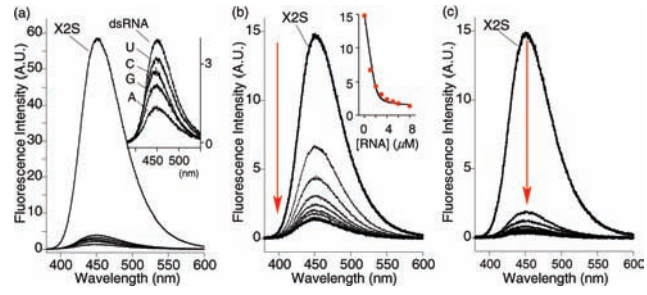

Figure 2. (a) Fluorescence spectra of X2S (10 μM) with N-bulge (N = U, C, G, A) and double-stranded RNA (N = none) (30 μM) in 10 mM sodium cacodylate (pH 7.0) and 100 mM NaCl. Inset: an enlarged plot. (b) Fluorescence spectra of X2S (2 μM) titrated with U-bulge RNA (0, 1, 2, 3, 4, 5, 6, and 8 μM). Inset: nonlinear least-squares fit (solid line) of the titration data at 453 nm to the 1:1 binding isotherm ($R = 0.98$). (c) Fluorescence spectra of X2S titrated with RRE under the same conditions as in (b).

saturation at a neomycin B concentration of 28 μM. In contrast, a peptide corresponding to amino acids 147−158 of the B-chain of human thrombin[12] did not induce fluorescence at all (Figure 3c), indicating that it does not compete with X2S in binding to RRE. Only modest fluorescence recovery was observed when the Rev peptide was added to the preformed complex of U-bulge RNA and X2S (Figure S4). The plot of fluorescence intensity against the concentration of the competitive ligands provides useful information on ligand binding (Figure 3c). The distinct sigmoidal increase in the fluorescence of the displacement assay with the Rev peptide suggests that cooperative binding of Rev to RRE displaces X2S-occupied Rev binding sites. The slow but steady increase in fluorescence in the presence of neomycin suggests that neomycin binding is not competitive with X2S binding at low concentrations. It has been reported that the neomycin high-affinity site in RRE is not competitive with Rev binding.[13]

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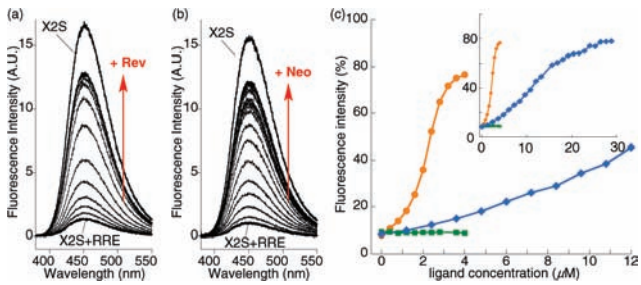

Figure 3. Displacement assay of the Rev−RRE interaction using X2S as the fluorescent indicator. The preformed X2S−RRE complex [2 μM in 10 mM sodium cacodylate (pH 7.0) and 100 mM NaCl] was displaced with increasing concentration of (a) Rev peptide (0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, and 4.0 μM) and (b) neomycin B (0−28.8 μM with a 1.2 μM increment). (c) Plots of relative fluorescence intensity ($F_{[obs]}/F_{[free\ X2S]}$, %) at 453 nm against ligand concentration. Key: Rev, ●; neomycin B, ◆; thrombin, ■. Inset: an expanded plot.

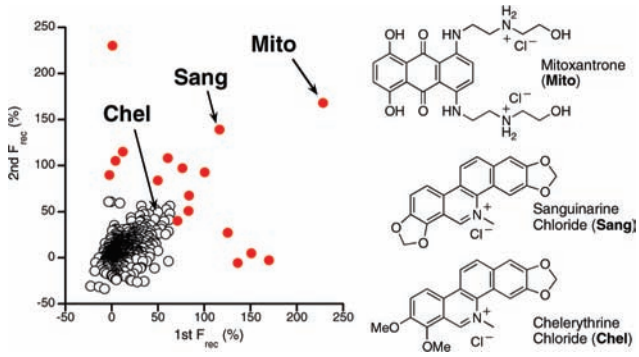

Figure 4. Displacement assay of the LOPAC1280 library against RRE with X2S as the fluorescent indicator. The preformed X2S-RRE complex [0.5 μM in 10 mM sodium cacodylate (pH 7.0) and 100 mM NaCl] was displaced with a compound (10 μM) in the LOPAC1280 library. Normalized fluorescence recovery (F_{rec}) values were calculated as $F_{rec} = 100\% \times (F_{+RRE+ligand} - F_{+RRE})/(F_{X2S} - F_{+RRE})$.

To validate the assay, ligands that bind to RRE were identified using the LOPAC1280 chemical library. A solution containing RRE (0.5 μM) and X2S (0.5 μM) in 10 mM sodium cacodylate (pH 7.0) containing 0.1% dimethyl sulfoxide and 100 mM NaCl was placed in 96-well assay plates, and the fluorescence of the solution was measured using a microplate reader in the absence (F_{+RRE}) or presence ($F_{+RRE+ligand}$) of the library compound (10 μM). The 59 compounds that fluoresced at a concentration of 10 μM were not used in the assay. The library compounds were evaluated using two independent assays. The results shown in Figure 4 were evaluated according to their normalized fluorescence recovery (F_{rec}) using the equation listed in the Figure 4 caption. Although most of the compounds had F_{rec} values less than 50%, 17 compounds that had $F_{rec} > 65\%$ in one of two assays were identified as hit compounds (indicated in red in Figure 4). The F_{rec} exceeded 100% in some cases because the fluorescence of the released X2S in the presence of the test drugs (10 μM) and RNA was greater than that of X2S in buffer alone (F_{X2S}). The F_{rec} threshold of 65% for identifying hit compounds was chosen to include all potential compounds that bind to RRE. Mitoxantrone (**Mito**) and sanguinarine chloride (**Sang**) had large F_{rec} values with good reproducibility. It is noteworthy that chelerythrine chloride (**Chel**), in which one of the benzodioxolane groups of **Sang** is replaced by two dimethoxy groups, had an F_{rec} reproducibility of only 50%, suggesting that **Sang** binds more strongly to RRE than **Chel**. Furthermore, isothermal titration calorimetry (ITC) analyses showed that exothermic heats of 6 and 5 μcal/mol were generated during the first additions of X2S and **Sang**, respectively, to the RRE solution, whereas the first addition of **Chel** resulted in the generation of only 1 μcal/mol (Figure S5). The response to **Chel** was as weak as that observed at the end of the **Sang** titration, suggesting that heat generation in the presence of **Chel** is likely caused by a nonspecific interaction. We also confirmed that the X2S displacement assay provided a different set of hit compounds with another RNA structure of HIV-1 mRNA (data not shown).

The X2S fluorescence indicator displacement assay is useful for qualitative evaluation of ligand−RNA interactions. The results of the assay are consistent with those of ITC analysis. The assay is suitable for high-throughput analysis of large chemical libraries against identified RNA targets.

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Supporting Information Available: Synthetic protocol for X2S, fluorescence spectra of X2S titrated with hairpin RNA, displacement assay of X2S−U-bulge RNA with Rev, ITC data for the interaction of ligands with RRE, fluorescence quenching of X2S with nucleotide monophosphates, and displacement assay for LOPAC1280. This material is available free of charge via the Internet at http://pubs.acs.org.

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