Biophysical Characterization of the Cocaine Binding Pocket in the Serotonin Transporter Using a Fluorescent Cocaine Analogue as a Molecular Reporter*

Received for publication, September 4, 2000, and in revised form, November 1, 2000
Published, JBC Papers in Press, November 2, 2000, DOI 10.1074/jbc.M008067200

Søren G. F. Rasmussen‡, F. Ivy Carroll§, Martin J. Maresch§, Anne Dam Jensen‡, Christopher G. Tate¶, and Ulrik Gether‡‡

From the ‡Division of Cellular and Molecular Physiology, Department of Medical Physiology, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark, §Chemistry and Life Sciences, Research Triangle Institute, Research Triangle Park, North Carolina, 27709, and the ¶Medical Research Council Laboratory of Molecular Biology, Cambridge, CB2 2QH United Kingdom

To explore the biophysical properties of the binding site for cocaine and related compounds in the serotonin transporter SERT, a high affinity cocaine analogue (3β-(4-methylphenyl)tropane-2β-carboxylic acid N-(N-methyl-N-(4-nitrobenzo-2-oxa-1,3-diazol-7-yl)ethanolamine ester hydrochloride (RTI-233); 50 = 14 nM) that contained the environmentally sensitive fluorescent moiety 7-nitrobenzo-2-oxa-1,3-diazole (NBD) was synthesized. Specific binding of RTI-233 to the rat serotonin transporter, purified from SF-9 insect cells, was demonstrated by the competitive inhibition of fluorescence using excess serotonin, citalopram, or RTI-55 (3β-(4-iodophenyl)tropane). Moreover, specific binding was evidenced by measurement of steady-state fluorescence anisotropy, showing constrained mobility of bound RTI-233 relative to RTI-233 free in solution. The fluorescence of bound RTI-233 displayed an emission maximum (λmax) of 532 nm, corresponding to a 4-nm blue shift as compared with the λmax of RTI-233 in aqueous solution and corresponding to the λmax of RTI-233 in 80% dioxane. Collisional quenching experiments revealed that the aqueous quencher potassium iodide was able to quench the fluorescence of RTI-233 in the binding pocket (kq = 1.7 M⁻¹), although not to the same extent as free RTI-233 (kq = 7.2 M⁻¹). Conversely, the hydrophobic quencher 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) quenched the fluorescence of bound RTI-233 more efficiently than free RTI-233. These data are consistent with a highly hydrophobic microenvironment in the binding pocket for cocaine-like uptake inhibitors. However, in contrast to what has been observed for small-molecule binding sites in, for example, G protein-coupled receptors, the bound cocaine analogue was still accessible for aqueous quenching and, thus, partially exposed to solvent.

Cocaine is one of the most widely abused psychostimulants, causing major medical and socioeconomic problems (1). Currently, there is no effective treatment against cocaine addiction available; therefore, clarifying the molecular mechanisms underlying the psychostimulatory effects and addictive properties of cocaine should prove critical for potential development of future therapeutic strategies. Cocaine and related drugs act by inhibiting clearance of released monoamine neurotransmitters from the synaptic cleft (2–4). This clearance of monoamines occurs via three distinct but highly homologous monoamine transporters, the serotonin transporter (SERT),1 the dopamine transporter (DAT), and the noradrenaline transporter (NET) (2–4). Cocaine binds with high affinity to all three transporters and is generally believed to act as a competitive blocker of substrate translocation (4, 5). Several studies have provided evidence that inhibition of the DAT is the predominant mechanism behind the stimulatory effects and addictive properties of cocaine (6–8). However, this hypothesis has been challenged by recent studies on mice in which the DAT gene has been deleted (1). Despite the absence of the DAT gene, it was surprisingly observed that these mice self-administered cocaine, indicating a possible important role of also the SERT and NET (9, 10).

The SERT belongs together with DAT and NET to a family of Na⁺/Cl⁻–dependent solute carriers that are characterized functionally by their dependence on the presence of Na⁺ and Cl⁻ in the extracellular fluid (3, 11). All Na⁺/Cl⁻–dependent carriers are believed to share a common topology characterized by the presence of 12 transmembrane segments connected by alternating extracellular and intracellular loops with an intracellular location of the N and C terminus (3, 4, 11). Despite intense efforts, including many mutagenesis studies (12–18) and studies using photoaffinity labeling (19), surprisingly little is known about the binding site for cocaine-like substances in the monoamine transporters. Although cysteine-scanning mutagenesis of transmembrane segment 3 in the SERT has suggested that two residues (Ile-172 and Tyr-176) in the middle of the transmembrane segment could be in close proximity to the cocaine binding site (20), no direct contact sites have been established between cocaine and specific transporter residues.

* The study was supported by the Danish Natural Science Research Council, National Institutes of Health Grants R01 DA12408 and R01 DA05477, the Lundbeck Foundation, and the NOVO Nordisk Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of an Ole Rømer Associate Research Professorship from the Danish Natural Science Research Council, National Institutes of Health Grants R01 DA12408 and R01 DA05477, the Lundbeck Foundation, and the NOVO Nordisk Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: SERT, serotonin transporter; rSERT, rat SERT; NET, noradrenaline transporter; DAT, dopamine transporter; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; 5-HT, 5-hydroxytryptamine; TEMPO: 2,2,6,6-tetramethylpiperidine-N-oxyl; RTI55, 3β-(4-iodophenyl)tropane; RTI-233, 3β-(4-methylphenyl)tropane-2β-carboxylic acid N-(N-methyl-N-(4-nitrobenzo-2-oxa-1,3-diazol-7-yl)ethanolamine ester.
Hence, it is not yet clear whether the cocaine binding site is deeply buried inside the transmembrane core of the transporter molecule or if the binding site is partially or fully exposed on the transporter surface.

In this study we have investigated the biophysical nature of the cocaine binding site in the rat SERT (rSERT). For this purpose, a cocaine analogue, which contained the environmentally sensitive fluorescent moiety, nitrobenzoxadiazol, were synthesized (see Fig. 1). Moreover, an epitope-tagged version of the rSERT was expressed in SF-9 insect cells and purified to obtain a transporter preparation that provided a sufficiently high signal-to-noise ratio for characterizing the fluorescent properties of the ligand bound to the rSERT. Our subsequent spectroscopic analysis of the bound fluorescent ligand provided evidence for a highly hydrophobic binding pocket. However, collisional quenching experiments showed that, despite the hydrophobic character of the binding crevice, the bound cocaine analogues were still accessible for aqueous quenching consistent with a partially exposed binding site. These findings contrast the observations for small-molecule ligand binding sites in other membrane proteins, such as, for example, G protein-coupled receptors, where the binding sites for small-molecule ligands are known to be deeply embedded in the transmembrane core of the molecule and entirely inaccessible to aqueous quenching (21, 22).

**Experimental Procedures**

**Synthesis of 3β-(4-Methylphenyl)tropane-2β-carboxylic Acid N-(N-methyl-N-(4-nitrobenzo-2-oxa-1,3-diazol-7-yl)ethanolamine Ester Hydrochloride (RTI-233)—** Oxalyl chloride (2.0 M in CH₂Cl₂; 0.80 ml, 1.60 mmol) was added dropwise to a stirred solution of 3β-(4-methylphenyl)tropane-2β-carboxylic acid (RTI-374) (23) (200 mg, 0.77 mol) in CH₂Cl₂ (20 ml) under an argon atmosphere at 25 °C. After stirring for 1 h, the CH₂Cl₂ was removed by reduced pressure. A solution of the acid chloride and N-methyl-N-(4-nitrobenzo-2-oxa-1,3-diazol-7-yl)aminooethanol (458 mg, 1.93 mmol) in CH₂Cl₂ (10 ml) under argon was treated slowly with triethylamine (0.5 ml, 3.5 mmol) and stirred at 25 °C for 38 h. Removal of CH₂Cl₂ by rotary evaporation afforded an orange-red residue, which was purified by column chromatography on silica gel (4.5 × 18 cm) with an ethyl acetate/methyl alcohol gradient elution (100:90-50:50 v/v) of the cell membrane or the purified protein. The purity of the cocaine analogue was then assessed by analytical reverse-phase high-performance liquid chromatography (HPLC) using a Chiralcel OJ-H column (5 μm, 250 × 4.6 mm) eluted with an isocratic mixture of 80% H₂O and 20% CH₂Cl₂ containing 0.1% HCl at a flow rate of 1 ml/min. The purity of the cocaine analogue was determined by monitoring the UV absorbance at 270 nm.

**Expression of the SERT in SF-9 Insect Cells—** The rSERT, tagged at C terminus with a myc epitope and at the C terminus with a 10× His epitope, was expressed in Sf-9 insect cells, and baculovirus encoding the tagged rSERT was expressed in Sf-9 insect cells, and baculovirus encoding the tagged rSERT, which stabilizes the transporter at the cell surface (24, 25).

**Membrane Preparation from SF-9 Insect Cells—** Membrane preparation from SF-9 insect cells was carried out by homogenizing the cells in digitonin buffer (25 mM Hepes, pH 7.5, containing 0.1% digitonin). The lysate was centrifuged at 40,000g for 30 min at 4 °C. The supernatant containing the solubilized transporter was purified by nickel chromatography using chelating Sepharose (Amersham Pharmacia Biotech). Binding to the resin was carried out in batch for 2 h at room temperature before separation of bound from unbound on 2 ml of Sephadex G-50 columns (Amersham Pharmacia Biotech). The purified transporter was concentrated using Centricon-30 spin concentrators (Amicon, Beverly, MA). The specific activity of the purified transporter was measured in a total volume of 125I-RTI-55 binding assay kit (Amersham Pharmacia Biotech). The purified transporter was assayed in a total volume of 125I-RTI-55 binding activity assay kit (Bio-Rad). The purified transporter was assayed in a total volume of 125I-RTI-55 binding activity assay kit (Bio-Rad). The purified transporter was assayed in a total volume of 125I-RTI-55 binding activity assay kit (Bio-Rad). The purified transporter was assayed in a total volume of 125I-RTI-55 binding activity assay kit (Bio-Rad).

**Fluorescence Spectroscopy—** Fluorescence spectroscopy was performed on a SPEX Fluoromax-2 fluorometer connected to a PC equipped with the DataMax 2.2 software package (Jobin Yvon Inc., Edison, NJ). In all experiments, the excitation and emission bandpass.
were set at 5 nm. For the emission scan, quenching, and anisotropy experiments, 20 pmol of purified rSERT was incubated in 100 µl of digitonin buffer (25 mM HEPES, pH 7.5, with 0.1% digitonin, 100 mM NaCl) in the presence of 1 µM RTI-233 and, when indicated, 1 mM 5-HT, 10 µM RTI 55, or 10 µM cetopram for 30 min at 4 °C before separation of bound from unbound on 2 ml of Sephadex G-50 columns. The fraction of bound was obtained by eluting with 1000 µl of ice-cold digitonin buffer. A 400-µl sample of the eluate was transferred to a 5 × 5-mm quartz cuvette (Helma, Mulheim, Germany) for the subsequent spectroscopic measurements. The absorption by the nonfluorescent ligands, 5-HT, cetopram, and RTI-55 is less than 0.01 at the used concentrations excluding any “inner filter” effect.

**Emission Scan Experiments**—The emission scans were performed either on 400 µl of Sephadex G-50-separated samples obtained as described above or directly on buffer/dioxane samples containing RTI-233. Excitation was 480 nm, and emission was measured from 495 to 625 nm with an integration time of 0.3 s/nm. All emission spectra are averages of three consecutive scans. Photobleaching was negligible under the experimental conditions used. The emission spectra were corrected for any background fluorescence by routinely subtracting control spectra on buffer alone.

**Collisional Quenching Experiments**—Stock solutions (1.0 µM) of the hydrophilic quencher potassium iodide containing 10 mM Na2S2O3 were prepared freshly for each round of experiments. The hydrophobic quencher 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) was dissolved in 10% Me2SO at a concentration of 100 mM and immediately used. The experiments were performed on either 400 µl of the Sephadex G-50-separated samples and prepared as described above or directly on buffer samples containing RTI-233. To correct for dilution/ionic strength effects on fluorescence, measurements were performed in parallel using a 1.0 x stock of potassium chloride (KCI) and a 10% Me2SO stock for the potassium iodide-and TEMPO-quenching experiments, respectively. Ten µl of quencher (potassium iodide or TEMPO) or control solution (potassium chloride or 10% Me2SO) was added sequentially followed by thorough mixing after each addition and subsequent recording of fluorescence using the Constant Wavelength Analysis program in the Data-max software package. The excitation wavelength was 480 nm, and the emission wavelength was either 532 nm for the recordings on RTI-233 bound to rSERT or 536 nm for the recordings on RTI-233 alone in digitonin buffer. A complete experiment was performed in 8 min, during which dissociation of ligand was negligible. In the experiments with TEMPO, the fluorescence intensities were corrected as described (26) for inner filter effects caused by the absorption by TEMPO at the used excitation and emission wavelengths. The corrected data were plotted according to the Stern-Volmer equation, F/F₀ = 1 + Ksv[Q], where F/F₀ is the ratio of fluorescence intensity in the absence and presence of quencher (Q), and Ksv is the Stern-Volmer quenching constant (27).

**Fluorescence Anisotropy**—The SPEX Fluoromax-2 fluorometer was equipped with an automated L-format polarization accessory including two Glan-Thomson UV polarizers placed in the sample chamber to enable polarized excitation and emission detection. The anisotropy measurements were carried out using the Constant Wavelength Analysis program with the excitation set at 480 nm and emission measured at 532 nm for RTI-233 bound to rSERT and 536 nm for free RTI-233 in digitonin buffer (integration time 10 s). Concurrent measurements of the emission intensity with the excitation-side polarizer in the vertical position (V) and the emission-side polarizer in either the V or horizontal position (H) were carried out. The measurements were collected at 4, 20, and 37 °C by perfusion of water with adequate temperature through the thermostatted cuvette holder. The data were converted to anisotropy according to the equation, A = (Iₐᵥ - GIₐᵥ)/(Iₐᵥ + 2GIₐᵥ), where Iₐᵥ is the intensity measured with both the excitation-side and emission-side polarizer in the vertical position (V), Iₐᵥ is the intensity measured with the excitation-side polarizer in the vertical position (V) and the emission-side polarizer in the horizontal position (H), and G is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light (Sᵥ/Sₜ) (27). The anisotropy was stable for at least 15 min at the indicated temperatures, indicating negligible dissociation of ligand under the experimental conditions used.

**RESULTS**

**Synthesis of a Fluorescent Cocaine Analogue**—To obtain a fluorescent analogue of cocaine with preserved high affinity for the rSERT, the environmentally sensitive fluorescent moiety 7-nitrobenzo-2-oxa-1,3-diazone (NBD) was connected to the 2β position of the tropane backbone in RTI-374 (3β-(4-methylphenyl)tropane-2β-carboxylic acid) using N-methylmethylaniline as the linker as described under “Experimental Procedures” (Fig. 1) (5). Importantly, very large 2β-carboxalkoxy groups have been shown to be rather well tolerated in both cocaine and in the 3-phenylopane analogue, RTI-55 (5). Notably, the fluorescent compound, RTI-233, differed, like RTI-55 and the parent 2β-carbomethoxy compound RTI-374 (3β-(4-methylphenyl)tropane-2β-carboxylic acid), from cocaine by having the aromatic ring directly connected to the 3 position of the tropane ring (Fig. 1). The 3-phenylopane-type of structure was chosen since it is known to increase binding affinity as compared with that of cocaine itself (5). As shown in Fig. 2, RTI-233 bound with high affinity to the rSERT expressed in Sf-9 cell membranes displaying a Kᵦ value of 6 nM for inhibition of 125I-RTI-55 binding (Fig. 2 and Table I). In comparison, the structurally related compound, RTI-55, and cocaine itself displayed Kᵦ values of 0.22 and 109 nM, respectively, for inhibition of 125I-RTI-55 binding.

**Binding of RTI-233 to the Purified rSERT**—The rSERT, expressed in Sf-9 insect cells, was purified by nickel chromatography followed by concanavalin A chromatography. The purified transporter bound RTI-233 with an affinity that was almost identical to that measured in the Sf-9 cell membranes (Fig. 2 and Table I). The Kᵦ for RTI-233 was 14 nM for the purified versus 6 nM for the transporter in membranes, as determined from competition binding assays with 125I-RTI-55 (Fig. 2 and Table I). Similarly, the Kᵦ values for serotonin and the two blockers RTI-55 and cetopram for inhibition of 125I-RTI-233 binding are almost identical to those measured in the Sf-9 cell membranes (Fig. 2 and Table I).

**Biophysical Characterization of the Cocaine Binding Pocket**

![Cocaine](image1.png)

**Fig. 1. Synthesis of RTI-233.** The cocaine analogue RTI-233 was synthesized as described under “Experimental Procedures” by connecting the environmentally sensitive fluorescent moiety NBD to the 2β position of the 3-phenylopane backbone using an N-methylmethylaniline linker. RTI-233 differs, like RTI-55, from cocaine by having the phenyl ring directly connected to the 3-position of the tropane ring.
RTI-55 binding to the purified transporter were similar to those observed in the membranes (Fig. 2 and Table I). These data suggest that the overall conformation of the transporter is conserved upon purification and support the contention that the purified rSERT in detergent micelles can be used as a model system for exploring the biophysical characteristics of the binding site for cocaine-like blockers.

**Spectral Properties of the Fluorescent Cocaine Analogue**

The emission from RTI-233 is highly sensitive to the polarity of the environment. In water, the \( \lambda_{\text{max}} \) (wavelength at which maximum emission occurs) of RTI-233 was 543 nm when the optimal excitation wavelength of 480 nm was used (Fig. 3A and Table II). Decreasing the polarity of the solvent by adding increasing concentrations of dioxane caused a significant blue shift in \( \lambda_{\text{max}} \) was 528 nm, and the fluorescence quantum yield was more than 5-fold higher than in water (Fig. 3A and Table II). Notably, in buffer containing 0.1% digitonin, the \( \lambda_{\text{max}} \) was 536 nm, consistent with a lowered polarity due to the presence of the detergent molecules (Table II). The strong dependence of the fluorescence from RTI-233 on the polarity of the surrounding solvent corroborated the possibility of using RTI-233 as molecular reporter of the microenvironment in the cocaine binding crevice of the rSERT.

**Evidence for a Highly Hydrophobic Microenvironment in the Binding Site for Cocaine-like Blockers**

To characterize the spectral properties of RTI-233 bound to the transporter, 1 \( \mu \)M RTI-233 was preincubated with purified rSERT (200 nM) for 30 min at 4 °C in the absence or presence of an excess of serotonin, citalopram, or RTI-55. Bound ligand was separated from unbound by rapid gel filtration using 2-ml G-50 gel filtration columns as described under “Experimental Procedures.” Subsequent fluorescence spectroscopy analysis revealed a strong fluorescence signal if no competing nonfluorescent ligand was present during precincubation (Fig. 3B). However, if the transporter was preincubated with RTI-233 together with an excess of either serotonin, citalopram, or RTI-55, the measured fluorescence was negligible (illustrated by a representative curve

![Figure 2](image2.png)

**Fig. 2.** Pharmacological profile of purified rSERT compared with rSERT in Sf-9 cell membranes. Competition binding experiments were performed on Sf-9 cell membranes (A) and on the purified transporter (B) as described under “Experimental Procedures.” Binding of \( ^{125}\text{I}-\text{RTI-55} \) (0.25 nM) to the transporter were competed with unlabelled RTI-55 (closed squares), RTI-233 (open squares), cocaine (closed circles), 5-HT (open circles), and citalopram (open triangles). Data are percentage of maximum \( ^{125}\text{I}-\text{RTI-55} \) binding (means \( \pm \) S.E., \( n = 3 \)).

![Figure 3](image3.png)

**Fig. 3.** Fluorescent properties of RTI-233 free in solution and bound to the purified rSERT. A, emission spectra of free RTI-233 in dioxane/H_2O. The fraction of dioxane ranges from 0 to 90% (v/v) with 10% intervals. B, emission spectrum of RTI-233 bound to rSERT (\( \lambda_{\text{max}} = 532 \) nm) with control emission spectra obtained by preincubation with an excess of 5-HT, citalopram, or RTI-55. The spectra shown are representative of nine experiments. In all experiments the excitation wavelength was 480 nm, with a 5-nm bandpass for both excitation and emission.

**Table I**

| rSERT in Sf-9 membranes | K_d [S.E. interval] | K_i [S.E. interval] | Hill slope | Purified rSERT |
|-------------------------|-----------------|-----------------|-------------|----------------|
|                         | \( \lambda_{\text{nm}} \) | \( \lambda_{\text{nm}} \) |               | \( \lambda_{\text{nm}} \) | \( \lambda_{\text{nm}} \) | Hill slope |
| RTI-55                  | 0.22 [0.187–0.260] |                 | -1.05       | 0.84 [0.771–0.915] |                 | -1.05       |
| 5-HT                    | 1112 [931–1237]   |                 | -1.09       | 441 [406–479]     |                 | -0.99       |
| Cocaine                 | 109 [106.8–112.0] |                 | -1.02       | 200 [194.0–205.6] |                 | -0.98       |
| RTI-233                 | 6.0 [5.06–7.04]   |                 | -1.16       | 13.7 [12.80–14.60] |                 | -0.93       |
| Citalopram              | 1.0 [0.96–1.05]   |                 | -1.01       | 2.4 [2.11–2.69]   |                 | -1.06       |

**TABLE I**

**Binding properties of purified rSERT in comparison to rSERT in Sf-9 membranes**

Competition binding on rSERT expressed in Sf-9 insect cell membranes and on purified transporter using 0.25 nM \( ^{125}\text{I}-\text{RTI-55} \) as radioligand. Values are from three independent experiments performed in triplicates. The \( K_d \) values were calculated by the equation \( K_d = IC_{50} - [\text{radioligand}] \). The IC_{50} values used for calculation of \( K_d \) values were obtained from means of pIC_{50} values determined by nonlinear regression analysis using Prism from GraphPad software (San Diego, CA) and the S.E. interval from pIC_{50} \( \pm \) S.E. \( K_d \) values were calculated according to the equation \( K_d = IC_{50}/(1 + L/K_d) \) where \( L \) is concentration of radioligand.
The means were determined as described under “Experimental Procedures.” Data for RTI-233 bound to the transporter at 4, 20, and 37 °C. The anisotropy of RTI-233 in water or digitonin buffer and of free RTI-233 in 80% dioxane (Fig. 3).

Fluorescence anisotropy of RTI-233 in water or digitonin buffer, and bound to rSERT are shown. The fluorescence intensities relative to water at \( \lambda_{\text{max}} \) are indicated for the water/dioxane mixtures. The emission maxima for RTI-233 in digitonin buffer and bound to rSERT are means ± S.E. of the indicated number of experiments.

| Emission maximum \( \lambda_{\text{max}} \) of RTI-233 | Relative fluorescence intensity at \( \lambda_{\text{max}} \) |
|---------------------------------|---------------------------------|
| H$_2$O                           | 543                             |
| 10% dioxane                      | 541                             |
| 20% dioxane                      | 540                             |
| 30% dioxane                      | 539                             |
| 40% dioxane                      | 538                             |
| 50% dioxane                      | 536                             |
| 60% dioxane                      | 535                             |
| 70% dioxane                      | 533                             |
| 80% dioxane                      | 532                             |
| 90% dioxane                      | 528                             |
| Digitonin buffer                 | 535.5 ± 0.49 (n = 6)            |
| Bound to rSERT                   | 531.6 ± 0.53 (n = 9)            |

in Fig. 3B). These data are consistent with the ability of these three structurally distinct compounds to compete with RTI-233 for the rSERT and supports that the spectrum obtained in the absence of these compounds is derived from RTI-233 specifically bound to the rSERT. As shown in Fig. 3B, this emission spectrum of bound RTI-233 displayed a \( \lambda_{\text{max}} \) of 532 nm, representing a 4-nm blue shift as compared with that observed for RTI-233 free in the buffer (Table II). According to the emission spectra of RTI-233, in different concentrations of the hydrophobic solvent dioxane, a corresponding blue shift was observed for free RTI-233 in 80% dioxane (Fig. 3A and Table II).

Evidence for Decreased Rotational Freedom of RTI-233 upon Binding to the rSERT—Anisotropy represents a measure of molecular motions at a nanosecond time scale (27). Accordingly, steady-state anisotropy measurements were carried out to investigate the rotational freedom of unbound RTI-233 as compared with RTI-233 bound to the rSERT (Fig. 4). The anisotropy of unbound RTI-233 in water was 0.057 ± 0.002 (means ± S.E., n = 3) at 20 °C, consistent with a substantial rotational freedom. In digitonin buffer, the anisotropy of unbound RTI-233 increased to 0.173 ± 0.002 (means ± S.E., n = 3) at 20 °C (Fig. 4). This increase reflects most likely some degree of interaction of the ligand with the detergent molecules, causing inhibition of the rotational freedom. Importantly, however, the anisotropy was further increased upon binding of RTI-233 to the rSERT (0.329 ± 0.004 at 20 °C; means ± S.E., n = 3; Fig. 4), indicating a significantly constrained rotational freedom of RTI-233 when bound to the transporter as compared with free in solution. As expected, the anisotropy was temperature-dependent, consistent with increasing rotational mobility at increasing temperatures (Fig. 4). The anisotropy was stable for more than 15 min at 20 °C, indicating negligible dissociation of the ligand under the experimental conditions used.

Probing Ligand Accessibility by Collisional Quenching—The accessibility of bound RTI-233, as compared with free RTI-233, was evaluated in collisional quenching experiments. Collisional quenching requires a bimolecular interaction between the quencher and the fluorophore, and therefore, such experiments can determine the “availability” of the fluorophore to the surrounding solvent (27). The aqueous quencher iodide (I$^-$) was found to be a strong quencher of the fluorescence from RTI-233 free in buffer. This is illustrated by the linear Stern-Volmer plot and a \( K_{SV} \) value of 1.7 M$^{-1}$ for the free ligand. The iodide quenching of bound RTI-233 was substantially smaller but still apparent with a linear Stern-Volmer plot and a \( K_{SV} \) value of 1.7 M$^{-1}$ (Fig. 5A). The accessibility of RTI-233 was further examined by comparing quenching produced by the lipid-soluble, nitroxide radical compound, TEMPO. The addition of increasing concentrations of TEMPO efficiently quenched both unbound and bound RTI-233. However, as shown in Fig. 5B, TEMPO was a stronger quencher of bound RTI-233 than of free RTI-233. Whereas the \( K_{SV} \) value was 37 m$^{-1}$ for the bound ligand, it was reduced to 16 m$^{-1}$ for...
the unbound ligand (Fig. 5F). It is important to note that the addition of potassium iodide up to a concentration of 200 mM and TEMPO up to a concentration of 20 mM did not affect binding of RTI-55 to the purified transporter (data not shown).

**DISCUSSION**

Although cocaine is one of the most widely abused psycho-stimulants, only little is known about the molecular mechanisms underlying the inhibitory effect of cocaine at the monoamine transporters (SERT, DAT, and NET). Most remarkable, the binding site for cocaine and related analogues in the transporters is still unknown. In this study, we have obtained new insight into the biophysical nature of this binding site for cocaine-like blockers using fluorescence spectroscopy techniques. To carry out the studies, we developed a fluorescent 3-phenyltropane (cocaine-like) analogue (RTI-233) that retained high affinity for the SERT despite the incorporation of the fluorescent NBD moiety. The NBD moiety is characterized by a high sensitivity of the emission to the polarity of the surrounding solvent, allowing the possibility of using the ligand as reporter of the biophysical environment in the blocker binding pocket. Initially, we tried to perform the spectroscopic measurements on Sf-9 cell membranes expressing the SERT; however, the nonspecific background fluorescence was too high to detect specific binding of RTI-233 (data not shown).

Therefore, a purification scheme for SERT expressed in Sf-9 cells was developed. The purified SERT displayed the same pharmacological profile as that observed in Sf-9 cell membranes (Fig. 2 and Table I). Furthermore, this pharmacological profile is similar to that observed for rSERT both in brain tissue (29, 30) and in transfected mammalian cell lines (13, 14, 31). This suggests that the overall structure of the SERT is conserved upon purification and, thus, that the purified preparation can be used as an appropriate model system for exploring the binding site for cocaine-like blockers as well as for studying other structure-function relationships in SERT. We should note that it cannot be excluded that the absence of a transmembrane ion gradient and the use of temperatures lower than 37 °C in our experiments to some extent could affect the conformation of the binding site. However, we have no indication that such effects are of any significance. Hence, the $\lambda_{\text{max}}$ for RTI-233 bound to the transporter is the same at 20 °C and at 37 °C, suggesting that the microenvironment is not changing upon changes in temperature (data not shown).

Several observations supported a highly hydrophobic microenvironment in the binding pocket for cocaine-like blockers. First, it was observed that the emission spectrum of RTI-233 bound to the SERT was similar to that for the free ligand in 80% dioxane. Second, the bound ligand was quenched substantially stronger by the lipid-soluble nitroxide compound TEMPO, than the free ligand. Third, the accessibility of bound RTI-233 to the aqueous quencher iodide was markedly decreased in comparison to free RTI-233. A noteworthy observation was, nevertheless, that RTI-233 bound to rSERT could still be quenched to some degree by the aqueous quencher iodide. It is interesting to compare this observation for a small-molecule inhibitor binding site in a transporter protein with data obtained for small-molecule ligands in other membrane proteins, such as G protein-coupled receptors. Notably, similar studies of inhibitor binding sites have not been carried before in Na$^+$-coupled transporter proteins. In the G protein-coupled $\beta_2$-adrenergic receptor, the fluorescent antagonist carazolol was used to probe the biophysical properties of the ligand binding pocket (21). The fluorescence emission spectrum of the bound carazolol was consistent with an extremely hydrophobic environment in the binding site of the receptor, and exposure to collisional quenchers demonstrated that carazolol bound to the purified $\beta_2$-adrenergic receptor was not accessible to the solvent at all (21). Furthermore, the fluorescence of bound carazolol was not quenched by exposure to sodium nitrite, a Forster energy acceptor having an $R_0$ value of 11.7 Å with carazolol (21). It was concluded, therefore, that the antagonist binds to the $\beta_2$-adrenergic receptor in a rigid hydrophobic environment buried deep within the transmembrane core of the protein (21). Similar results were obtained for a fluorescently derivatized small-molecule nonpeptide antagonist of the NK-1 (substance P) receptor (22). The fluorescence emission of the bound nonpeptide antagonist was not sensitive to aqueous quenchers, consistent with a deeply buried binding site similar to that of carazolol in the $\beta_2$-adrenergic receptor (22). The spectral properties of the fluorescent nonpeptide antagonist were compared with those of a fluorescently derivatized substance P analogue.

In contrast to what was observed for the nonpeptide compound, the fluorescence from this fluorescent peptide bond to the receptor was readily quenched by aqueous quenchers, consistent with the predicted binding of the larger peptide ligands on the extracellular face of the receptors (22). Taken together, our current data differ from the observations for small ligands in G protein-coupled receptors in that the binding site for cocaine-like blockers is only partially buried and, thus, cannot be deeply embedded in the protein interior. This conclusion is interesting in light of the recent finding that cocaine can protect Cys-135 and Cys-342 in the first and third intracellular loop from reaction with the sulfhydryl-reactive methanethiosulfonate reagent, MTSEA (2-Aminethyl methanethiosulfonate) (32). It was concluded that the protection by cocaine was due to an indirect conformational effect; however, it is intriguing to consider the possibility that the two cysteines in the intracellular loops are in close proximity to the cocaine binding site of the monoamine transporters.

Many previous studies have aimed at defining the binding site for cocaine and other blockers in the monoamine transporters. Generation of chimeric transporters have been used to identify the domain important for the selective recognition of, for example, tricyclic anti-depressants, but due to lack of cocaine selectivity among the monoamine transporters, chimeric studies have provided little insight into determinants of cocaine binding (33, 34). Surprisingly, substitution of multiple single residues has provided only limited additional information about residues important for recognition of cocaine-like substances. Nonconservative substitutions of a series of prolines and phenylalanines caused only minor changes in the affinities for the cocaine analogue WIN 35,528, making it highly difficult to assess whether these changes are due to direct effects or indirect structural perturbations (17, 18). Similarly, mutation of an aspartic acid in the cytoplasmic half of TM 1 (Asp-98), which is conserved among the monoamine transporters and believed to be critical for substrate recognition, only marginally affected cocaine affinity (14). It did, however, cause a more than 100-fold decrease in the apparent affinity of citalopram, suggesting a pivotal role of this residue in binding this inhibitor (14). Additional residues have been implicated in binding of other inhibitors but not cocaine-like substances. These include Tyr-95 in TM 1 and Phe-596 in TM 12 that were found to dictate the species selectivity of mazindol and imipramine between the human and rat transporter. However, substitution of these residues hardly affected cocaine binding affinity (13, 35). Rudnick and co-workers (20) recently carried out a systematic cysteine mutagenesis scan of TM3 and identified two residues that could be in proximity to the cocaine binding pocket. This conclusion was based on the observation that derivatization of cysteines inserted in these two positions...
Biophysical Characterization of the Cocaine Binding Pocket

4723

(Ile-172 and Tyr-176) with the positively charged methanethiosulfonate reagent, MTSET ([2-(Trimethylammonium)ethyl]methanethiosulfonate), severely inhibited binding of the cocaine analogue, 2β-carbomethoxy-3β-4-[125I]iodophenyl)tropane (20). Moreover, this inhibition was blocked by cocaine itself (20). It was nonetheless difficult to assess whether the observed effects were direct or indirect; hence, it is clear that specific interactions between cocaine and individual residues in the transporters remains to be identified. Although we have to take into consideration that the RTI-233 and cocaine binding pockets may not be entirely identical, the current data could be an indication that the further search for direct cocaine interactions should not be restricted to residues that are deeply embedded in the predicted core of the transporter molecule but may also include residues closer to the transporter surface.

In summary, we have carried out a biophysical characterization of the binding site for cocaine-like blockers in the rSERT. For this purpose we have synthesized a fluorescent cocaine analogue that, to the best of our knowledge, is the first fluorescent cocaine analogue displaying high affinity for the rSERT. Furthermore we have developed a purification scheme for the rSERT expressed in Sf-9 insect cells. In addition, to provide the first direct information about the microenvironment of the binding pocket for cocaine-like blockers, the fluorescent ligand together with the purification procedure should prove important tools in future studies. Such studies could include, for example, further mapping of the binding site for cocaine-like blockers based on measurements of fluorescent resonance energy transfer (FRET) between the bound ligand and cysteine-reactive fluorescent probes site-selectively incorporated into the purified transporter molecule.

Acknowledgment—Dr. Brian Kobilka is thanked for helpful comments on the manuscript.

REFERENCES

1. Caine, S. B. (1998) Nat. Neurosci. 1, 90–92
2. Horn, A. S. (1990) Prog. Neurobiol. 34, 387–400
3. Girov, B., and Caron, M. G. (1993) Trends Pharmacol. Sci. 14, 43–49
4. Povlock, S. L., and Amara, S. G. (1997) in Neurotransmitter Transporters: Structure, Function, and Regulation (Reith, M. E. A., ed) pp. 1–28, Humana Press Inc., Totowa, NJ
5. Carroll, F. I., Lewin, A. H., and Kuhar, M. J. (1997) in Neurotransmitter Transporters: Structure, Function, and Regulation (Reith, M. E. A., ed) pp. 263–295, Humana Press Inc., Totowa, NJ
6. Ritz, M. C., Lamb, R. J., Goldberg, S. R., and Kuhar, M. J. (1987) Science 237, 1219–1223
7. Girov, B., Jaber, M., Jones, S. R., Wightman, R. M., and Caron, M. G. (1996) Nature 379, 606–612
8. Wisse, R. A. (1996) Annu. Rev. Neurosci. 19, 319–340
9. Rocha, B. A., Fumagalli, F., Gainetdinov, R. R., Jones, S. R., Ato, R., Girov, B., Miller, G. W., and Caron, M. G. (1998) Nat. Neurosci. 1, 132–137
10. Xu, F., Gainetdinov, R. R., Wetsel, W. C., Jones, S. R., Bohn, L. M., Miller, G. W., Wang, Y. M., and Caron, M. G. (2000) Nat. Neurosci. 3, 465–471
11. Nelson, N. (1998) J. Neurochem. 71, 1785–1803
12. Kitayama, S., Shimada, S., Xu, H., Markham, L., Donovan, D. M., and Uhl, G. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7782–7785
13. Barker, E. L., Perlman, M. A., Adkins, E. M., Houlihan, W. J., Pristupa, Z. B., Niznik, H. B., and Blakely, R. D. (1998) J. Biol. Chem. 273, 19459–19468
14. Barker, E. L., Moore, K. R., Rakhshan, F., and Blakely, R. D. (1999) J. Neurosci. 19, 4705–4717
15. Norregaard, L., Frederiksen, D., Nielsen, E. O., and Gether, U. (1998) EMBO J. 17, 4266–4273
16. Loland, C. J., Norregaard, L., and Gether, U. (1999) J. Biol. Chem. 274, 36928–36934
17. Itokawa, M., Lin, Z., Cai, N. S., Wu, C., Kitayama, S., Wang, J. B., and Uhl, G. R. (2000) Mol. Pharmacol. 57, 1093–1103
18. Lin, Z., Itokawa, M., and Uhl, G. R. (2000) FASEB J. 14, 715–728
19. Vaughn, R. A. (1995) Mol. Pharmacol. 47, 956–964
20. Chen, J. G., Sachatzidis, A., and Rudnick, G. (1997) J. Biol. Chem. 272, 28321–28327
21. Tota, R. T., and Strader, C. D. (1990) J. Biol. Chem. 265, 16891–16897
22. Turecatt, G., Zoffmann, S., Lowe, J. A., Ill, Drozdz, E. S., Chassais, G., Schwartz, T. W., and Chollet, A. (1997) J. Biol. Chem. 272, 21167–21175
23. Carroll, F. I., Kotian, P., Dehghani, A., Gray, J. L., Kuzemko, M. A., Parham, K. A., Abraham, P., Lewin, A. H., Boja, J. W., and Kuhar, M. J. (1995) J. Med. Chem. 38, 379–388
24. Tate, C. G., Whiteley, E., and Betenbaugh, M. J. (1999) J. Biol. Chem. 274, 17551–17558
25. Tate, C. G., and Blakely, R. D. (1994) J. Biol. Chem. 269, 26303–26310
26. London, E. (1986) Annu. Rev. Biochem. 54, 57–63
27. Lakowics, J. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 238–318, Kluwer Academic Publishers/Plenum Publishing Group, New York
28. Gether, U., Lin, S., and Kobilka, B. K. (1995) J. Biol. Chem. 270, 28268–28275
29. Boja, J. W., Mitchell, W. M., Patel, A., Kopajtic, T. A., Carroll, F. I., Lewis, A. H., Abraham, P., and Kuhar, M. J. (1992) J. Pharmacol. Exp. Ther. 262, 21167–21175
30. Rothman, R. B., Cadet, J. L., Akunne, H. C., Silverthorn, M. L., Baumann, M. H., Carroll, P. I., Rice, K. C., de Costa, B. R., Partilla, J. S., and Wang, J. B. (1994) J. Pharmacol. Exp. Ther. 270, 296–309
31. Eshleman, A. J., Carmolli, M., Cymbay, M., Martens, C. R., Neve, K. A., and Janowsky, A. (1999) J. Pharmacol. Exp. Ther. 290, 877–885
32. Ferrer, J., and Javitch, J. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9238–9243
33. Buck, K. J., and Amara, S. G. (1995) Mol. Pharmacol. 48, 1030–1037
34. Girov, B., Wang, Y. M., Suter, S., McLeskey, S. B., Pilli, C., and Caron, M. G. (1994) J. Biol. Chem. 269, 15985–15988
35. Barker, E. L., and Blakely, R. D. (1998) Methods Enzymol. 296, 475–498