The Human Dopamine Transporter Forms a Tetramer in the Plasma Membrane

CROSS-LINKING OF A CYSTEINE IN THE FOURTH TRANSMEMBRANE SEGMENT IS SENSITIVE TO COCAINE ANALOGS*

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Using cysteine cross-linking, we demonstrated previously that the dopamine transporter (DAT) is at least a homodimer, with the extracellular end of transmembrane segment (TM) 6 at a symmetrical dimer interface. We have now explored the possibility that DAT exists as a higher order oligomer in the plasma membrane. Cysteine cross-linking of wild type DAT resulted in bands on SDS-PAGE consistent with dimer, trimer, and tetramer, suggesting that DAT forms a tetramer in the plasma membrane. A cysteine-depleted DAT (CD-DAT) into which only Cys243 or Cys306 was reintroduced was cross-linked to dimer, suggesting that these endogenous cysteines in TM4 and TM6, respectively, were cross-linked at a symmetrical dimer interface. Reintroduction of both Cys243 and Cys306 into CD-DAT led to a pattern of cross-linking indistinguishable from that of wild type, with dimer, trimer, and tetramer bands. This indicated that the TM4 interface and the TM6 interface are distinct and further suggested that DAT may exist in the plasma membrane as a dimer of dimers, with two symmetrical homodimer interfaces. The cocaine analog MFZ 2–12 and other DAT inhibitors, including benztrapine and mazindol, protected Cys243 against cross-linking. In contrast, two substrates of DAT, dopamine and tyramine, did not significantly impact cross-linking. We propose that the impairment of cross-linking produced by the inhibitors results from a conformational change at the TM4 interface, further demonstrating that these compounds are not neutral blockers but by themselves have effects on the structure of the transporter.

The dopamine transporter (DAT) is responsible for the re-uptake of dopamine released into the synaptic cleft and thus represents the major mechanism for the termination of dopaminergic neurotransmission (1). DAT is a member of the family of Na+/Cl− and Cl−-dependent neurotransmitter transporters, which includes transporters for other neurotransmitters, including norepinephrine, serotonin, γ-aminobutyric acid, and glycine, as well as a number of other small molecules (2, 3). The family also includes a large number of homologous bacterial and archaeal proteins, one of which has been recently identified as a Na+−dependent tryptophan transporter (4). These transporters couple the movement of sodium down its concentration gradient to the transport of substrate, but the molecular mechanism of this process is understood only in general terms. The Na+− and Cl−-dependent neurotransmitter transporters are thought to have 12 transmembrane segments with intracellular NH2 and COOH termini, and this general topology has been supported by the accessibility of putative extracellular and intracellular loop residues to chemical modification (5–8). Although the packing of the 12 TMs is unknown, some distance constraints have been established in DAT through the identification of an endogenous zinc binding site and subsequent use of engineered metal binding sites (9, 10).

The serotonin transporter (SERT) was inferred to be a homooligomer, based on co-immunoprecipitation studies of differentially epitope-tagged SERT constructs and on the functional effects of chemical modification of co-expressed SERT mutants (11). In addition, cross-linking of SERT was also consistent with an oligomeric structure of SERT, although the residues responsible for cross-linking were not identified (12). The demonstration of fluorescence resonance energy transfer between different spectral variants of green fluorescent proteins fused to the NH2 termini of SERT, GAT (13), and more recently DAT (14), also supports their existence as dimers. In contrast, Horiuchi et al. (15) inferred from their studies that mature glycine transporter in the plasma membrane was not oligomeric, whereas immature intracellular transporter was oligomeric.

Using cysteine cross-linking, we demonstrated that DAT is at least a homodimer, with the extracellular end of TM6 at a symmetrical dimer interface (16). Moreover, we identified the presence of the dimerization motif GVGVGXXVX(T/A) (17, 18) in TM6 of DAT and of many related neurotransmitter transporters, and we engineered an inhibitory Zn2+-binding site at the extracellular end of TM6 near the dimer interface (19). Recently, using different approaches, studies in both DAT (20) and GAT (21) have supported the existence of a dimer interface involving TM2. Whether the TM2 interface, which in both of these studies was disrupted by mutations in a leucine heptad repeat, is at the same homodimer interface as the TM6 interface or whether DAT is a higher order oligomer with distinct interfaces is unknown. Using cysteine cross-linking, we have now explored the possibility that DAT exists as a higher order oligomer in the plasma membrane. We show that DAT appears to be a tetramer in the plasma membrane and that TM4 is present at a homo-dimeric interface distinct from the TM6

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interface. Moreover, we show that a dopamine uptake inhibitor inhibits cross-linking of a cysteine in TM4, suggesting the presence of a conformational change at the TM4 interface upon inhibitor binding.

EXPERIMENTAL PROCEDURES
Stable Transfection of DAT—EM4 cells (human embryonic kidney 293 cells stably transfected with macrophage scavenger receptor to increase their adherence to tissue culture plastic (22)) were stably transfected with a synthetic human DAT gene (synDAT) that was tagged at the amino terminus with a FLAG epitope fused to the hemagglutinin epitope with the first 22 amino acids truncated (FLAG-HA) as described previously (16). Some experiments were also conducted with a full-length synDAT tagged at the NH₂ terminus with a FLAG epitope as described previously (23).

Cross-linking—Cross-linking was performed for 5 min at room temperature as described previously (16) with 100 μM CuSO₄ and 400 μM 1,10-phenanthroline (CuP), 20 μM bis-EA, 20 μM HgCl₂, or 1 mM CuSO₄ (Cu²⁺). Pretreatment with 1 μM MFZ 2–12, 10 μM mazindol, 10 μM benzotripine, 100 μM dopamine, or 100 μM tyramine was performed, when indicated, for 20 min at room temperature before cross-linking, which was performed in the continued presence of these compounds. After cross-linking, the cells were washed and DAT extracted for immunoblotting as described previously (16).

Immunoblotting—Samples were applied to 1.5-mm, 15-well 7.5% acrylamide gels. For quantitation of the apparent molecular mass of the cross-linked DAT, gels were stained with Coomassie Brilliant Blue R-250 and visualized by photography. Immunoblotting was performed as described previously (16), and chemiluminescence was detected and quantitated on a FluorChem 8000 (Alpha Innotech Corp.).

RESULTS AND DISCUSSION
We demonstrated previously that DAT heterologously expressed in HEK 293 cells was cross-linked to a dimer by copper phenanthroline (CuP) and that Cys306 at the extracellular end of TM6 was the endogenous cysteine that is cross-linked at a symmetrical homodimer interface (16). Consistent with these findings, we also observed CuP-induced cross-linking of DAT to dimer in mouse striatal membranes (16). In addition to dimer, however, in mouse striatal membranes we also demonstrated the appearance of a larger oligomeric species containing DAT, suggesting that DAT might exist as a higher order oligomer but that a difference in the cellular context might somehow limit additional CuP-induced cross-linking in the HEK 293 background.

To explore this phenomenon further, we evaluated a number of other cross-linking reagents with FLAG-tagged wild type DAT expressed in HEK 293 cells. CuSO₄ (Cu²⁺) has been used in the absence of phenanthroline to cross-link cysteines in G-actin (24), and we found that although CuP and bis-EA only produced DAT dimer, treatment with Cu²⁺ produced cross-linked DAT species consistent with dimer, trimer, and tetramer at 177 ± 4, 269 ± 6, and 341 ± 6 kDa, respectively, with some residual monomer at 84 ± 2 kDa (mean ± S.E., n = 5). Identical results were observed with FLAG-HA-DAT in which the 22 NH₂-terminal residues are truncated (Fig. 1A). When expressed as a fraction of the total immunoreactivity in the lane, the percentage of monomer, dimer, trimer, and tetramer was 33 ± 5, 41 ± 4, 15 ± 2, and 12 ± 3, respectively (mean ± S.E., n = 5). In contrast, a cysteine-depleted DAT (CD-DAT) (16) (in which the only cysteines are Cys180 and Cys189, which likely form a native disulfide bond (25, 26), and Cys243 and Cys245, which are in cytoplasmic loops (5)) was not cross-linked by Cu²⁺ (Fig. 1A), despite the fact that it is expressed at the plasma membrane and functional (16). Substitution of the endogenous Cys306 back into CD-DAT resulted in the appearance of a dimer band in response to Cu²⁺ treatment (Fig. 1B) just as we observed with CuP (16). Thus, Cys306 is cross-linked by both CuP and Cu²⁺.

A FLAG-HA DAT in which Cys90 and Cys306, the two cysteines inferred to be accessible from the extracellular milieu (5), were mutated to Ala was inefficiently cross-linked to dimer by Cu²⁺ (Fig. 1B), although we previously showed that this construct was not cross-linked at all by CuP (16). Therefore, an endogenous cysteine or cysteines present in C90A/C306A, but absent in CD-DAT, must be cross-linked by Cu²⁺ but not by CuP. Mutation of Cys243 to Ala (C243A) in the C90A/C306A background completely diminished the Cu²⁺-induced cross-linking (Fig. 1B). This implicated Cys243 in TM4 as the cross-linked residue, and consistent with this hypothesis, CD-DAT with only Cys243 restored was cross-linked to dimer by Cu²⁺, albeit inefficiently (Fig. 2A). In addition, CD-DAT containing both Cys243 and Cys306 was cross-linked to dimer, trimer, and tetramer in a manner indistinguishable from that of wild type DAT (Fig. 2B).

Cross-linking of Cys243 by Cu²⁺ was inefficient when compared with that of Cys306 (Figs. 1B and 2A). Moreover, in contrast to Cys306 no cross-linking of Cys243 was observed with CuP or with bis-2-aminomethylmethanethiosulfonate (bis-MTSEA) (Fig. 3). Reasoning that this might have resulted from poor access of the cross-linking reagents to a protein interface surrounded by lipid, we attempted cross-linking with HgCl₂, which has been used to cross-link membrane-embedded cysteines (27). HgCl₂ cross-linked Cys243 much more efficiently than did Cu²⁺ (compare Fig. 3 and 2A). The size of the cross-linked species, ~176 kDa, was consistent with it being a homodimer of DAT. HgCl₂ cross-linked Cys306 as well, with optimal cross-linking seen at lower concentrations (data not shown). Importantly, the CD-DAT background was not cross-linked at all by HgCl₂ (Fig. 3).

It was not possible to study the effect of cross-linking on uptake because HgCl₂ potently inhibited uptake by CD-DAT (data not shown), presumably due to reaction with cytoplasmic Cys342, the modification of which inhibits uptake (28). Cu²⁺ also potently inhibited uptake by CD-DAT (data not shown), consistent with the presence of an inhibitory Cu²⁺ binding site (29). We were able, however, to assess whether bound ligand impacted cross-linking of Cys243. The potent cocaine analog MFZ 2–12 (30) dramatically decreased mercury-induced cross-linking of CD-DAT-A243C (Fig. 4B). In addition, the uptake inhibitors benzotripine and mazindol also protected against cross-linking, whereas the substrates dopamine and tyramine did not significantly affect cross-linking (Fig. 4B).

2 Cocaine protected against the weak cross-linking of Cys243 induced by Cu²⁺, but in preliminary experiments cocaine protected less efficiently against mercury-induced cross-linking (data not shown). These differences may relate to the very high reactivity of mercury and/or the lower affinity and faster dissociation rate of cocaine relative to the other inhibitors tested.
CuP-induced cross-linking of Cys306 at the extracellular end of TM6, was unaffected by MFZ 2–12 (data not shown). Consistent with protection against cross-linking of Cys243 but not Cys306, the trimer and tetramer bands produced by Cu2+ were eliminated when cross-linking was performed in the presence of MFZ 2–12, leaving only the dimeric species in which Cys306 was cross-linked (Fig. 4A).

If TM4 and TM6 were situated at the same homo-dimer interface, then cross-linking would be expected to produce only dimer. The appearance of dimer, trimer, and tetramer, therefore, is consistent with the existence of two distinct homodimer interfaces. Thus, DAT appears to exist in the membrane as a dimer of dimers with distinct symmetrical interfaces involving both TM4 and TM6. This is consistent with our earlier observation of higher order oligomeric species upon cross-linking of DAT in mouse striatal membranes (16) and also with a radiation inactivation study in which DAT was inferred to be a tetramer (31). Experiments with concateners of SERT also supported a dimeric or tetrameric, but not a trimeric, quaternary structure (32). Inactivation studies with coexpressed SERT mutants were also interpreted as consistent with SERT possibly being a tetramer (11).

Unlike Cys306, Cys243 was not cross-linked by CuP or by bis-EA, suggesting that it is not readily accessible to the aqueous environment. Consistent with this, treatment with MTSEA did not prevent cross-linking of Cys243 by Cu2+ or by HgCl2 (data not shown), suggesting that Cys243 is not accessible to reaction with MTSEA. Cu2+ must gain limited access to the site, but cross-linking by Cu2+ is quite inefficient. Mercury is known to react rapidly and selectively with sulphydryl groups and can bridge nearby pairs of cysteines to form an intermolecular mercury-linked dimer (27). Mercury is able to enter a hydrophobic environment (27), which likely accounts for its more efficient cross-linking of Cys243.

In contrast to Cys306, the cross-linking of which was altered by cocaine-like inhibitors, cross-linking of Cys243 was dramatically inhibited by the cocaine analog MFZ 2–12, as well as by other structural diverse uptake inhibitors, including benztpine and mazindol (Fig. 4). This adds to the growing literature demonstrating that cocaine-like molecules and other uptake inhibitors are not simply neutral blockers but by themselves produce conformational rearrangements of DAT (5, 33, 34).

There are two potential explanations for the decreased cross-linking of Cys243 seen with uptake inhibitors. First, DAT might dissociate from a tetramer into a dimer upon inhibitor binding. In this scenario, the tetramer may be required for function, and dissociation of this oligomeric species may be a critical mechanism of inhibition of uptake as well as block of currents associated with cocaine (23, 35). Second, the tetrameric structure of...
DAT may be stable, but the inhibitors may cause a conformational change at the TM4 interface that decreases cross-linking. Based on preliminary cross-linking and protection data on an extensive series of TM4 cysteine substitution mutants,1,2 we think it most likely that a conformational change, rather than dissociation of the tetramer, is responsible for the protection against cross-linking. It is interesting to speculate whether the interfaces contribute to the transport pathway or ion conduction pathways in DAT and related transporters, and this will merit further investigation. It seems unlikely that inhibitors sterically block access of the cross-linkers to Cys243 given the lack of significant impact of mutation of Cys243 on binding efficiency of cross-linking of Cys243 makes the higher order sons, such as inefficient transfer, or because the relatively low porters, may somehow prevent DAT from forming an extended interface in a tetrameric complex, and ongoing studies attempting to cross-link cysteines in TM2 of DAT may help to resolve this as well.

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