

Zn²⁺ site engineering at the oligomeric interface of the dopamine transporter

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Abstract Increasing evidence suggests that Na⁺/Cl[−]-dependent neurotransmitter transporters exist as homo-oligomeric proteins. However, the functional implication of this oligomerization remains unclear. Here we demonstrate the engineering of a Zn²⁺ binding site at the predicted dimeric interface of the dopamine transporter (DAT) corresponding to the external end of transmembrane segment 6. Upon binding to this site, which involves a histidine inserted in position 310 (V310H) and the endogenous Cys306 within the same DAT molecule, Zn²⁺ potently inhibits [³H]dopamine uptake. These data provide indirect evidence that conformational changes critical for the translocation process may occur at the interface between two transporter molecules in the oligomeric structure. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Monoamine transporter; Na⁺/Cl[−]-dependent neurotransmitter transporter; Oligomerization; Dimerization; Metal ion binding site

1. Introduction

The dopamine transporter (DAT) terminates dopaminergic neurotransmission by mediating rapid reuptake of released dopamine from the synaptic cleft [1,2]. Together with the closely related transporters for norepinephrine (NET) and serotonin (SERT) the transporter belongs to the large family of Na⁺/Cl[−]-dependent transporters that are characterized by the presence of 12 putative transmembrane domains and intracellular N- and C-termini [1–3]. The packing of the 12 helices is poorly understood, and no high-resolution structural information is currently available.

The quaternary structure of Na⁺/Cl[−]-dependent neurotransmitter transporters also remains unknown. Interestingly, an increasing amount of evidence suggests that Na⁺/Cl[−]-dependent neurotransmitter transport proteins form homo-oligomeric structures although the functional significance has not been clarified. In the SERT, formation of oligomers was supported biochemically by the finding that SERTs tagged

with different epitopes can be co-immunoprecipitated [4]. The observation of fluorescence resonance energy transfer between different spectral variants of green fluorescence protein (YFP and CFP) fused to the SERT have also provided evidence for the existence of SERT oligomers in living cells [5]. In the DAT, we obtained data suggesting that transmembrane segment (TM) 6 forms part of a homodimeric interface [6]. Most importantly, we found that a cysteine situated at the extracellular end of TM 6 (Cys306) can be symmetrically cross-linked between two DAT molecules either through formation of a disulfide bridge catalyzed by copper phenanthroline or by covalent cross-linking using a bifunctional cysteine-reactive cross-linker [6].

In this study we have taken advantage of the strict geometrical requirements for binding of the Zn²⁺ ion and attempted to engineer Zn²⁺ binding sites at the predicted oligomeric interface of DAT at the extracellular end of TM 6 by performing a series of sequential histidine substitutions. Specifically, we wished to investigate whether structural constraints imposed by Zn²⁺ binding at the oligomeric interface either within the same transporter molecule or between two adjacent molecules could affect transporter function.

2. Materials and methods

2.1. Site-directed mutagenesis

The cDNA encoding the human dopamine transporter (hDAT) was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC, USA). Mutant transporters were constructed by polymerase chain reaction (PCR)-derived mutagenesis using *Pfu* polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The generated PCR fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis, and cloned into the eukaryotic expression vector, pRc/CMV (Invitrogen, Carlsbad, CA, USA), containing hDAT. All mutations were confirmed by restriction enzyme mapping and DNA sequencing.

2.2. Cell culture and transfection

COS-7 cells were maintained as described and transiently transfected by the calcium phosphate precipitation method [7,8].

2.3. [³H]Dopamine uptake experiments

Uptake assays were performed modified from Giros et al. [9] using 2,5,6-[³H]dopamine (6.5–60 Ci/mmol) (Amersham Pharmacia Biotech). Transfected COS-7 cells were plated in either 96 well dishes (0.25 × 10⁵ cells/well), 24 well dishes (10⁵ cells/well) or 12 well dishes (2–3 × 10⁵ cells/well) to achieve an uptake level of 5–10% of total added [³H]dopamine. The uptake assays were carried out 2 days after transfection. Prior to the experiment, the cells were washed once in 100/500 µl of uptake buffer (25 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1 mM L-ascorbic acid and

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Abbreviations: hDAT, human dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; WT, wild-type; TM, transmembrane segment

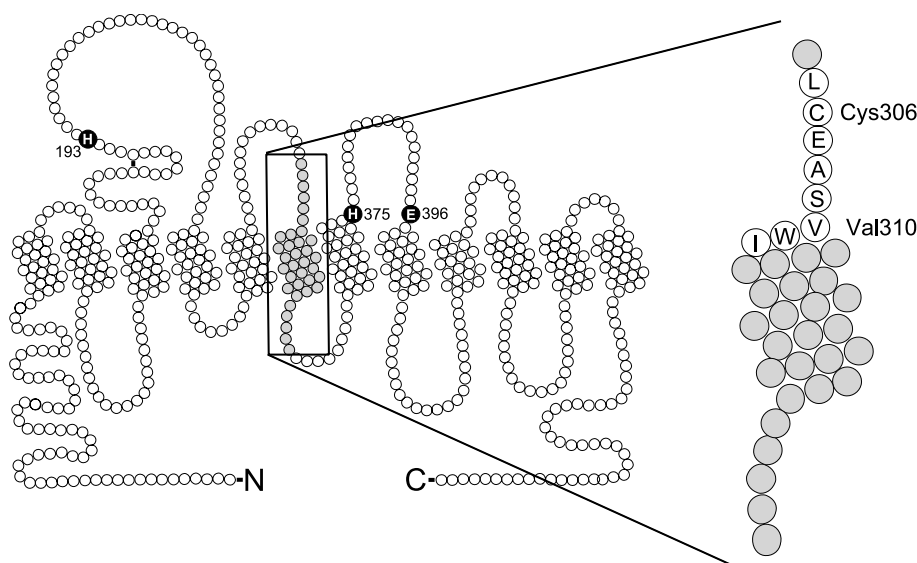


Fig. 1. Two-dimensional representation of hDAT. The three coordinating residues of the endogenous Zn^{2+} binding site of hDAT (His193, His375 and Glu396) are highlighted (white letters in enlarged black circles) [10,11]. To generate artificial Zn^{2+} binding sites in the region encompassing the external end of TM 6, His193 was mutated to lysine. The positions substituted with histidines at the extracellular end of TM 6 are indicated by black letters in enlarged white circles (residues 305–312).

5 mM D-glucose, pH 7.4). Unlabelled dopamine, RTI-55 and/or Zn^{2+} as indicated was added to the cells in varying concentrations, and uptake was initiated by addition of 10 nM [^3H]dopamine in a final volume of 100/500 μl . After 10 min incubation at 37°C , the cells were washed twice with 100/500 μl uptake buffer, lysed in 50/300 μl SDS and left 1 h at 37°C . 96 well samples were retained in their well whereas 24 and 12 well samples were transferred to 24 well counting plates (Wallac, Turku, Finland). 600 μl of Opti-phase Hi Safe 3 scintillation fluid (Wallac) was added followed by counting of the plates in a Wallac Tri-Lux β -scintillation counter (Wallac). Non-specific uptake was determined in the presence of 1 mM non-labeled dopamine (Research Biochemicals International, Natick, MA, USA). All determinations were performed in triplicate.

2.4. Cross-linking experiments

Cross-linking experiments were performed as described previously [6].

2.5. Calculations

Uptake data were analyzed by non-linear regression analysis using Prism 3.0 from GraphPad Software (San Diego, CA, USA).

3. Results

Zn^{2+} potently inhibits [^3H]dopamine uptake in a biphasic manner with a high affinity component ($\text{IC}_{50} = 0.49 \mu\text{M}$) and a low affinity component ($\text{IC}_{50} > 1000 \mu\text{M}$) in COS-7 cells transiently expressing the wild type (WT) hDAT (Table 1 and [10]). Whereas the low affinity component most likely is due to non-specific toxic effects of Zn^{2+} , the high affinity component is due to interaction of Zn^{2+} with an endogenous high affinity Zn^{2+} binding site consisting of three coordinating residues, His193 in the second extracellular loop, His375 at the top of TM 7 and Glu396 at the extracellular face of TM 8 [10,11]. By substituting His193 with lysine, which is present in the corresponding position in the NET, the Zn^{2+} potency is markedly reduced compared to hDAT WT (IC_{50} , 130 μM , Table 1, Fig. 2 and [10]). This mutant expresses similarly to the WT and was chosen as background for our attempts to engineer new Zn^{2+} binding sites at the predicted oligomeric interface at the top of TM 6.

Single histidine substitutions were made of residues 305–312 (Fig. 1 and Table 1). Substitution of Leu305 (H193K–L305H) resulted in a non-functional transporter with no detectable [^3H]dopamine uptake (Table 1). All other mutants were functional with K_M values for dopamine uptake ranging from 0.5 μM to 4 μM (Table 1). H193K–C306H, H193K–E307H, H193K–A308H, H193K–S309H, and H193K–I312H displayed V_{max} values that varied from 50% to 133% of that of the WT (2900–8000 fmol/min/ 10^5 cells). The V_{max} values for H193K–V310H and H193K–W311H were reduced to 400 and 100 fmol/min/ 10^5 cells, respectively (Table 1). In parallel to this observation, we were unable to detect specific binding of

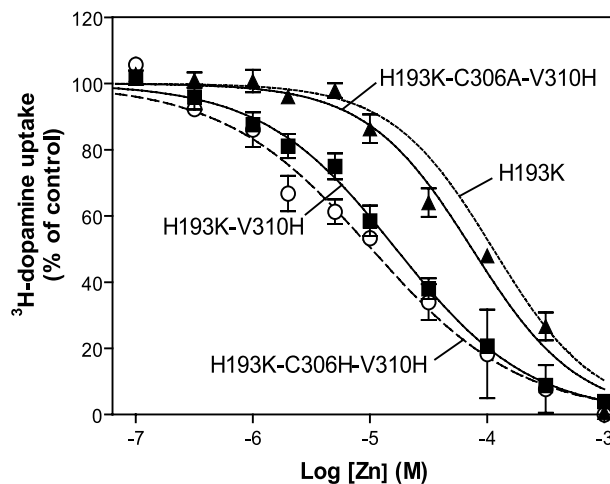


Fig. 2. Evidence for Zn^{2+} binding between a histidine in position 310 and Cys306 at the external face of TM 6 in hDAT. The figure shows Zn^{2+} inhibition of [^3H]dopamine uptake in COS-7 cells transiently expressing hDAT-H193K–V310H (black squares), hDAT-H193K–C306A–V310H (black triangles) or hDAT-H193K–C306H–V310H (white circles). The Zn^{2+} inhibition curve for hDAT-H193K is shown for comparison as a dotted line. Data are the means $\pm$ S.E.M. of three to seven experiments performed in triplicate.

Table 1
Dopamine uptake characteristics for hDAT WT and mutants

hDAT mutant	K_M μ M [S.E.M. interval]	V_{max} (fmol/min/ 10^5 cells) $\pm$ S.E.M.	n	IC_{50} (Zn^{2+}) μ M [S.E.M. interval]	n
WT	3.0 [2.4–3.7]	6000 ± 1200	8	0.49 [0.46–0.52]	6
H193K	2.3 [1.7–3.1]	6500 ± 900	9	130 [110–140]	7
H193K-L305H	N.D.	N.D.	2	N.D.	
H193K-C306H	4.0 [3–5]	8000 ± 600	5	190 [160–240]	4
H193K-E307H	2.6 [2.5–2.8]	7000 ± 100	5	370 [320–420]	4
H193K-A308H	4.0 [3–5]	6000 ± 100	5	140 [130–160]	4
H193K-S309H	2.35 [2.31–2.40]	7600 ± 200	5	90 [80–100]	4
H193K-V310H	0.8 [0.7–1.1]	400 ± 20	6	16 [10–24]	4
H193K-W311H	0.5 [0.3–0.6]	100 ± 10	3	320 [260–390]	2
H193K-I312H	0.6 [0.4–0.9]	2900 ± 1200	3	120 [90–160]	3

ND: not detectable. The K_M and V_{max} values for [3H]dopamine uptake were calculated from non-linear regression analysis of dopamine uptake data. Zn^{2+} inhibition of [3H]dopamine uptake was fitted to either a two site or a one site model. When fitted to the two site model, only the high affinity binding site is noted. The IC_{50} values used in the estimation of K_M were calculated from means of pIC_{50} values and the S.E.M. interval from the $pIC_{50} \pm$ S.E.M.

[^{125}I]RTI-55 in H193K–V310H and H193K–W311H whereas specific binding similar to the WT was observed in the other mutants (data not shown). Importantly, due to a lower sensitivity of the binding assay this could in fact be expected if the reduced V_{max} was due to lower expression of functional protein for these two mutants. The loss of [^{125}I]RTI-55 binding was not due to a decrease in affinity for RTI-55 since mutation of Val310 to histidine did not change the ability of RTI-55 to inhibit [3H]dopamine uptake in both constructs ($IC_{50} = 5$ [3–6] nM in H193K ($n=3$) as compared to 2.1 [1.9–2.4] nM ($n=3$) in H193K–V310H and 8 [6–12] nM in H193K–W311H ($n=2$), means followed by the S.E.M. interval).

All mutants were tested for their sensitivity to Zn^{2+} in [3H]dopamine uptake experiments. Only in one of the mutants (H193K–V310H) we observed a marked increase in apparent Zn^{2+} affinity as reflected in a decrease in IC_{50} value from 130 μ M in H193K to 16 μ M in H193K–V310H (Fig. 2 and Table 1). The IC_{50} values for Zn^{2+} remained > 90 μ M in the other mutants and thus similar to H193K. The apparent increase in affinity for Zn^{2+} in V310H suggests that a new Zn^{2+} binding site had been established in the transporter. First, we excluded the possibility that this new site involved any of the coordinating residues of the endogenous site at the top of TM 7 and 8 (His375 and Glu396) since mutation of these in the background of H193K–V310H did not alter the Zn^{2+} sensitivity of the transporter (data not shown). Next, we investigated the possibility that the nearby Cys306 was involved (besides histidines, only cysteines and acidic residues are capable of efficiently coordinating Zn^{2+} [12]). As shown in Fig. 2 and Table 2, substituting Cys306 with an alanine markedly decreased the potency of Zn^{2+} inhibition of [3H]dopamine uptake in H193K–C306A–V310H. Accordingly, the increased Zn^{2+} susceptibility seen in hDAT–H193K–V310H is likely due to the coordination of Zn^{2+} by at least Cys306 and His310 within the same DAT molecule. Additional support for this hypothesis

was obtained by the observation that a histidine could substitute for the cysteine in position 306 (hDAT–H193K–C306H–V310H) with respect to Zn^{2+} inhibition of [3H]dopamine uptake (Table 2 and Fig. 2). Furthermore, removing the histidine in position 310 from H193K–C306H–V310H resulting in H193K–C306H increased the IC_{50} for Zn^{2+} inhibition of dopamine uptake from 16 μ M to 190 μ M (Tables 1 and 2 and Fig. 2).

The molecular mechanism underlying the inhibitory effect of Zn^{2+} H193K–V310H was investigated by performing saturation [3H]dopamine uptake experiments in the presence and absence of 10 μ M Zn^{2+} . The experiments showed that Zn^{2+} was acting as a non-competitive inhibitor of substrate uptake in H193K–V310H, as the presence of 10 μ M Zn^{2+} did not change the K_M value for [3H]dopamine uptake ($K_M = 0.5$ [0.3–0.9] μ M in the presence of 10 μ M Zn^{2+} and 0.7 [0.4–1.0] μ M in the absence of Zn^{2+} , means of $n=3$ followed by the S.E.M. interval) whereas the level of substrate transport was reduced by more than 60% ($V_{max} = 200 \pm 40$ and $V_{max} = 530 \pm 130$ in the presence and absence of 10 μ M Zn^{2+} respectively, means of $n=3$ followed by S.E.M.). In comparison, neither the K_M value for [3H]dopamine uptake nor the uptake level was affected by 10 μ M Zn^{2+} in the control mutant H193K (data not shown).

Since Cys306 can be symmetrically cross-linked between two DAT molecules [6], it is potentially possible that positions 310 also are in close proximity across the dimeric interface of DAT. Accordingly, we carried out cross-linking studies in a DAT construct containing the mutations C306A–V310C. However, in this construct, under conditions that give robust cross-linking of Cys306, we saw no evidence of cross-linking of Cys310, either with copper phenanthroline or with a series of different length bifunctional methanethiosulfonate reagents (data not shown). Next we wished to test the possibility that the interaction responsible for the Zn^{2+} binding site between

Table 2
 Zn^{2+} inhibition of [3H]dopamine uptake in hDAT–H193K–V310H and control mutants

hDAT mutant	IC_{50} (Zn^{2+}) μ M [S.E.M. interval]	n
H193K–V310H	16 [10–24]	4
H193K–C306A–V310H	70 [60–90]	3
H193K–C306H–V310H	6 [3–12]	3
H193K–C306H	190 [160–240]	4

The Zn^{2+} inhibition data of [3H]dopamine uptake was fitted to either a two site or a one site model. When fitted to the two site model, only the high affinity binding site is noted. The IC_{50} values used in the estimation of K_M were calculated from means of pIC_{50} values and the S.E.M. interval from the $pIC_{50} \pm$ S.E.M.

positions 306 and 310 occurs across the dimer interface between Cys306 in one DAT and His310 in a second DAT. To do this, we attempted to cross-link between a Flag-tagged DAT construct containing the wild-type Cys306 and Val310, and a stably co-expressed Myc-tagged DAT construct with C306A–V310C. After treatment with copper phenanthroline and the bifunctional reagent bis-(2-methanethiosulfonatoethyl)amine hydrochloride we observed dimeric species of the Flag-tagged construct resulting from the cross-linking of Cys306 in two DATs as described previously [6], but failed to observe any Myc-tagged DAT in an oligomeric species (data not shown), indicating that Cys310 was not cross-linked to itself (consistent with the results described above) or with Cys306 in a second DAT molecule.

4. Discussion

A wide range of membrane proteins have been shown to exist as oligomeric complexes, e.g. receptor tyrosine kinases, voltage-dependent and ligand-gated ion channels as well as G protein-coupled receptors [13,14]. It is currently being discussed to what degree Na⁺-coupled transporters form oligomeric structures. For example, the Na⁺-glucose transporter-1 (SGLT-1) is most likely not an oligomer as evidenced by freeze-fracture electron microscopy [15]. In contrast, freeze-fracture studies of the glutamate transporter EAAT-3 suggested a putative pentameric structure of this transporter [16]. Among the Na⁺/Cl[−]-dependent neurotransmitter transporters, evidence against oligomerization has only been obtained for the glycine transporters (GLYT-1 and GLYT-2) [17] whereas convincing evidence suggests the existence of both DAT oligomers and SERT oligomers [4–6].

The functional importance of Na⁺/Cl[−]-dependent neurotransmitter transporter oligomerization is not known. If the oligomerization is important for the translocation process, it can be envisaged that conformational changes critical for the transport process occur at the dimeric interface and/or are transmitted between the subunits coupling the transporter subunits functionally together in the oligomeric complex. In this context, it is interesting to note that the crystal structures of the dimeric extracellular domain of a metabotropic glutamate receptor in agonist-bound and unbound states revealed disulfide-linked homodimers whose ‘active’ and ‘resting’ conformations are modulated through the dimeric interface by a packed α -helical structure [18]. The fact that we were unable to show an effect of Cys306 cross-linking [6] could argue against a similar scenario in the DAT. On the other hand, Cys306 may be situated in a flexible loop and accordingly disulfide formation between two adjacent Cys306 residues may not impose a structural constraint sufficient to inhibit conformational changes at the dimeric interface of the transporter. This may nonetheless be possible closer to or in the transmembrane region if the individual DAT molecules are functionally linked to one another.

In this study we have attempted to address this question by taking advantage of the strict geometrical requirements for binding of the small Zn²⁺ ion that have been thoroughly defined from crystal structures of numerous Zn²⁺ binding proteins [12]. Of particular importance for the present study, these structures have unravelled that the distance between the coordinating atom of the Zn²⁺ binding residue (i.e. a histidine, cysteine, aspartate, or glutamate) and the Zn²⁺ ion aver-

ages 2.0 Å [12]. Two residues involved in coordination of the same Zn²⁺ ion must therefore be situated in close spatial proximity in the tertiary or quaternary structure of the protein. We have previously utilized this information to probe tertiary structure relationships in the DAT. First, we identified three coordinating residues in a naturally occurring Zn²⁺ binding site and subsequently we were able to engineer a series of ‘non-natural’ Zn²⁺ binding sites in the transporter [10,11,19]. Upon binding to both the endogenous and the engineered sites, Zn²⁺ potently and non-competitively inhibited dopamine uptake [10,11,19]. Most likely, this occurred by constraining conformational changes critical for the translocation process [10,11,19].

To investigate whether structural constraints imposed by Zn²⁺ binding at the oligomeric interface could affect transporter function we made a series of histidine substitutions at the external end of TM 6 in the background of a mutant (H193K) insensitive to Zn²⁺ in micromolar concentrations. Our results suggested that Zn²⁺ could be coordinated between a histidine inserted in position 310 (V310H) and the endogenous Cys306. The selective ability of Cys306 and His310 positioned four residues apart to bind Zn²⁺ together with the apparent inability of Zn²⁺ to bind when histidines were inserted in the *i*+1 (G307H), *i*+2 (A308H), *i*+3 (S309H) or *i*+5 (W311H) position relative to Cys306 underlines the strict specificity of the Zn²⁺ interaction.

We recently showed that Cys306 can be symmetrically cross-linked between two DAT molecules [6]. Nonetheless, we find it most likely that Zn²⁺ is coordinated by His310 and Cys306 only in the same molecule of H193K–V310H. Zn²⁺ binding sites involving only two coordinating residues generally display an affinity around 10^{−5} M [19–21]. If both Cys306 and His310 coordinated Zn²⁺ in two adjacent molecules, a much higher affinity would be expected (10^{−8}–10^{−9}) [20–22]. Moreover, we were unable to cross-link a cysteine inserted in position 310 between two DAT molecules, suggesting that the side chains of the residues in position 310 may not be as close in the quaternary structure as those in position 306 and accordingly that Zn²⁺ coordination between His310 in two adjacent molecules is less likely. We were also unable to obtain experimental support for the alternative possibility that Zn²⁺ is being coordinated between Cys306 in one molecule and His310 in the adjacent molecule. Co-expression of H193K (containing Cys306) with H193K–C306A–V310H did not result in increased Zn²⁺ sensitivity in comparison to control as would be expected if Zn²⁺ was coordinated between two DAT molecules (data not shown). Moreover, as described in Section 3, we observed no cross-linking between Cys306 in one DAT and Cys310 in a differentially epitope-tagged co-expressed DAT.

In summary, we have been able to engineer a Zn²⁺ binding site at the external end of TM 6 corresponding to the predicted dimeric interface for the DAT. Binding of Zn²⁺ to this site was found to markedly impair transporter function. This underlines the functional importance of this region of the transporter and provides indirect evidence that conformational changes critical for the translocation process occur at the oligomeric interface between two DAT molecules. It is therefore tempting to speculate that oligomerization may have a direct functional role rather than being just a structural feature of putative importance for proper protein folding and protein stability.

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