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Novel structure-function information on biogenic amine transporters revealed by site-directed mutagenesis and alkylation

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Abstract

The study reported by Wenge and Bönisch in this issue provides critical structural information regarding extracellular loop 2 (EL2) of the human norepinephrine transporter (NET). A systematic search among all 10 cysteine and 13 histidine residues in NET led to His222 in EL2 as the target for N-ethylmaleimide: its alkylation interferes with [³H]nisoxetine binding, indicating the part of EL2 containing His 222 reaches back into the protein interior where it prevents access by nisoxetine to its binding site. Thus, EL2 in human NET does much more than conformationally assisting substrate translocation. The present study underscores the importance of site-directed mutagenesis approaches to elucidate structural features that cannot be deduced from crystals of homolog proteins. In the case of NET, the closest crystal structure is that of the homolog LeuT, but EL2 is difficult to align with 22 less loop residues in LeuT than in NET. The present results could only be achieved by the systematic mutagenesis study of all cysteines and all histidines in NET.

Keywords

biogenic amine transporters; structure-function; extracellular loops; extracellular vestibule S2; nisoxetine

Extensive cysteine scanning and chimera work by the Rudnick lab over the last one and a half decade on extracellular loops of the serotonin transporter (SERT) has indicated that rather than playing a role in substrate or drug binding, these loop regions are important for conformational changes required for substrate translocation by the protein [1-3]. The closest crystal available for homology modeling of biogenic amine transporters, the LeuT structure [4], has provided a wealth of structural guidance, but the extra- and intracellular loops deviate substantially between the LeuT and the mammalian biogenic amine transporters [5]. Especially extracellular loop 2 (EL2) of the latter is difficult to align with that of the LeuT which has 22, 21, or 16 amino acids less than the norepinephrine transporter (NET), the dopamine transporter (DAT), or SERT.

In this issue of *Neurochemical Research*, Wenge and Bönisch [6] describe a thorough structure-function study of the NET, asking the question which residue(s) is alkylated by N-ethylmaleimide (NEM) to reduce the binding of [³H]nisoxetine, a NET-specific radioligand. (The effect on substrate uptake could not be monitored due to NEM's effect on cellular ATP

required for active uptake.) After ruling out any of the ten NET cysteines as the target for NEM, Wenge and Bönisch went on to study all thirteen histidines, as histidine can also be alkylated by NEM. This systematic search led to one histidine residue being responsible for the irreversible inhibition by NEM of [³H]nisoxetine binding to NET: His222 in EL2, positioned at the C-terminal side of the likely α -helical segment of EL2 of ~ 11 amino acids. As mutation of His222 to Ala did not significantly alter [³H]nisoxetine binding, this residue is not directly involved in nisoxetine binding, but its alkylation by NEM sterically hinders binding. This suggests that the part of EL2 containing His 222 reaches back into the protein interior where it prevents access by nisoxetine to its binding site. In the LeuT crystal a different extracellular loop, EL4, is known to reach back into the protein, providing a lid to tricyclic binding in the extracellular vestibule, commonly referred to as the S2 site [7-9]. Mutation of Thr381 or Asp 473 in the S2 site in NET severely impacts nisoxetine's affinity [10]. This, along with (i) the idea advanced by Wenge and Bönisch [6] that the EL2 reaches into the mouth-like external cavity, and (ii) evidence for a distal binding of nisoxetine and the cocaine analog CFT, with the latter likely binding to the S1 site [11], is consonant with nisoxetine binding to the S2 site (see also the recent paper of Wang et al. [12]). The Wenge and Bönisch study [6] in this issue of *Neurochemical Research* provides exciting indication that EL2 in human NET does much more than conformationally assisting substrate translocation – the close proximity of the EL2 portion containing His222 to the binding site for nisoxetine indicates additional structural roles for EL2. His222 is conserved in the equivalent position in SERT and DAT, and thus EL2's role in these biogenic amine transporters may also be more prominent than previously thought.

The present study underscores the importance of site-directed mutagenesis approaches to elucidate structural features that cannot be deduced from crystals of homolog proteins. The present results could only be achieved by the systematic study of all cysteines and all histidines in NET, and comes from a laboratory that has been consistently in the forefront of transporter research, starting in the seventies with the pioneering work of kinetically characterizing neuronal uptake of norepinephrine. A truly impressive journey!

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