**Method 1: A suggested specific substrate-binding mode in a structural MCT8 homology model**

Propositions for amino acids participating in T3 binding based on functional data

To decipher potential localization of substrate recognition and binding sites in MCT8, iodothyronine T3 was docked into a new structural transporter homology model. The mode of T3 binding suggested in this study was generated based on a combination of known functional-structural information.

Presumptuously assuming similarities in the binding of a specific substrate to different proteins, we used a T3-receptor/T3 complex to define putative binding motifs for T3 in MCT8 in our previous study (Kleinau, et al. 2011). In brief, binding of T3 to the activated nuclear receptor can be observed between arginine and histidine at a particular distance to one another (around 15Å), whereby the middle portion of the T3 molecule is surrounded by hydrophobic / aromatic side-chains (Nascimento, et al. 2006). In the MCT8 homology model, such residue combinations (Arg445-His192, Arg301-His192 or Arg301-His415) were examined, which fulfill these distance parameters inside the putative substrate transport channel. Additionally, a further positively charged residue, Lys418, is located inside the transport channel, but was not crucially designated to interact with T3, due to non-preferred Lys418-His distances and potential steric hindrances. Different functional arguments for the potential significance of these aforementioned residues have been put forward:

1. Arginine 445 has been previously identified by site-directed mutagenesis studies and, as deduced from the occurrence of a pathogenic mutation (R445C), recognized to be important for substrate binding and translocation (Capri, et al. 2013; Kinne, et al. 2010). In addition, a dual role of this positively charged residue for substrate binding and conformational switching of MCT8 has been postulated (Kinne et al. 2010). Spatial localization of a functionally essential positively charged amino acid in the center of the transporter’s translocation channel is also known from other MFS (Franco, et al. 2006; Hagenbuch and Meier 2003; Sun, et al. 2012) and MCT members (Halestrap 2012, 2013; Manoharan, et al. 2006).

2. The recently reported pathogenic mutation H192R hampers substrate transport (Capri et al. 2013) and highlights position 192 as being important for substrate translocation, which is confirmed by the results of recent mutagenesis studies (Braun, et al. 2013; Groeneweg, et al. 2013). Thus, not only His192 but also His415 are significant for substrate translocation of MCT8 (Braun et al. 2013).

3. Arg301 is close to the intracellular site and on the basis of a mutagenesis approach, has been recently suggested to be potentially involved in substrate allocation (Braun et al. 2013).

Specification of molecular details for T3 binding in the MCT8 homology model

An additional protein-crystal structure bound with T3 was studied, in order to further elucidate this issue. Similar binding properties for T3 were also assumed for T3 and other proteins, which are evolutionary distant from one another and from MCT8. In particular, transthyretin (TTR) is an
extracellular transport protein that binds T3 and is involved in the allocation of thyroid hormones in
the body periphery. A crystal structure of a TTR/T3 complex is available (PDB code 1SN5, (Eneqvist,
et al. 2004)) and demonstrates, in similarity to the T3 transporter protein/T3 complex, that a positively
charged residue is important for substrate binding. However, in contrast to the T3 receptor, a
negatively charged side-chain of glutamate also participates in T3 binding at the carboxyl group
through H-bonding. The positively and negatively charged side-chains interact with one another in
order to position and fix the substrate. Therefore, we studied our refined MCT8 model primarily
focusing on negatively charged residues in close spatial distance to the previously suggested Lys/His
residues, with the aim of predicting TH binding. Indeed, Asp498 (TMH10), Glu422 (TMH7) and
Glu426 (TMH7) were revealed to be in spatial proximity to the positively charged side-chains of
Lys418 (TMH7) and Arg445 (TMH8), which was also found in MCT8. Strikingly, in other MCT
family members, negatively charged residues are observed at the corresponding MCT8 positions, i.e.
Asp498 in MCT10; Glu422 in MCT10, MCT11, and MCT13; Glu426 in MCT2, MCT5, MCT6,
MCT10, and MCT13. Lysine 418 is only conserved for MCT8 and MCT10, whereas Arg445 is
conserved amongst all MCT members (Kinne et al. 2010).

**T3 docking into the transport channel of MCT8**
The substrate T3 was pre-oriented manually inside the transport channel of the MCT8 homology
model, with an overall localization of its carboxyl-group between Arg445 and Lys418, while the
phenylic moiety of T3 was oriented between histidine side-chains 192 and 415 in the center of the
protein. The aim was to adjust the substrate by molecular dynamics allowing for negatively charged
side-chains, and finally to suggest interaction-combinations between the T3 carboxyl-group and
Arg/Lys-Glu/Asp motifs.

By fixing the backbone and distance constraints to 3Å between the side-chains of Arg445/Lys418 and
the T3 carboxyl group, molecular dynamics (2ns) were subjected to flexible amino acid side-chains of
the protein model and T3 (Sybyl-X, Certara). The entire system was then minimized until
convergence at a termination gradient of 0.05 kcal/mol*Å was reached. This procedure was repeated
thrice. Finally, the model was minimized without constraints using the AMBER 7.0 force field.

**Method 2: Measurements of T3 uptake**
The benefit of thyroid hormone uptake studies in COS-7 cells using hCRYM as an intracellular
binding protein of iodothyronines has been previously reported (Friesema, et al. 2008; Kersseboom, et
al. 2013). In our study, COS-7 cells were cultured in triplicates in 12-well plates (1 x 10^5 cells per
well) and transiently transfected with 250 ng N-HA-MCT8 constructs or empty pcDNA3 (mock) and
250 ng hCRYM (Abgent, US) subcloned in pcDNA3 expression vector using 1 µl Metafectene.
Seventy-two hours after transfection, cells were washed twice with 1x PBS. Samples were then
incubated for 60 s at room temperature with 4.9 nM non-radioactive T3 and 0.1 nM \(^{125}\text{I}\)T3 (Hartmann Analytic, Germany) in 500 µl assay buffer, i.e. 125 mM NaCl, 5 mM KCl, 1.3 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 25 mM HEPES, 5.6 mM Glucose; pH 7.4. Following incubation, the assay buffer was removed and the cells were washed twice with 1x PBS/0.1% BSA on ice. Finally, the cells were lysed with 40 nM NaOH and radioactivity as a measure of T3 uptake was counted for 60 s in a gamma-counter.
REFERENCES


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