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Thesis Paper

Optimization of experimental procedure for assessing transition metal ion FRET in LeuT †

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Received June 2017, Accepted April 2018

Neurotransmitter sodium symporters (NSSs) are important for the regulation of neurotransmitters, such as dopamine and serotonin, which are involved in addiction and depression, along with several other diseases. The bacterial NSS, LeuT, has been proven to be a good model protein for the mammalian NSSs. Conformational changes of LeuT can be examined using transition metal ion Förster Energy Resonance Transfer (tmFRET), where the energy transfer between a fluorescien dye and a di-histidine bound Ni^{2+} is measured. When utilizing tmFRET, free Ni^{2+} is added to the solution, which also adds to the unspecific quenching signal detected. It is therefore necessary to remove the signal from the unbound Ni^{2+}, in order to properly investigate the impact of specific bound Ni^{2+}. Here we investigate whether the effect of Zn^{2+} can be used to inhibit FRET contribution from specifically bound Ni^{2+} and thereby isolate the non-specific signal. We found that Zn^{2+} can competitively inhibit the binding of Ni^{2+} to the di-histidine motif of LeuT, thereby representing an easier, and perhaps more consistent, method for removing the signal from unbound Ni^{2+} during tmFRET measurements.

1 Introduction

Transport proteins are essential building blocks of life. They are among other things involved in transporting ions and small molecules, such as neurotransmitters or amino acids across biological membranes. The majority of known neurotransmitter transporters belong to the neurotransmitter sodium symporter (NSS) family [1]. NSSs transport their solute by utilizing the energy gained from the sodium gradient, which is created by the Na^+/K^+ ATPase. Furthermore, all mammalian NSS co-transport Cl^-. [1] Important members of this family include transporters of dopamine (DAT), noradrenaline (NET) and serotonin (SERT). [2] NSSs are involved in the removal of transmitters from the synapse, thereby terminating transmission from neurotransmitters. This involvement in the control of synaptic signaling has made NSSs key targets for therapeutics and illicit drugs, such as cocaine or amphetamines [3]. The regulation of neurotransmitters such as dopamine and serotonin are thereby involved in addiction, as well as depression and other diseases such as ADHD or schizophrenia [4]. This makes NSSs very interesting to examine. However, the mammalian transporters have proven exceptionally challenging to purify sufficiently, as satisfactory stability, yield and purity has been difficult to obtain in order to properly investigate their structure. The bacterial NSS, LeuT, from the Aquifex aeolicus has, however, proven to be much easier to purify. Furthermore, LeuT has been shown to be a suitable model protein, as a high degree of structural conservation has been observed between LeuT and the mammalian NSSs [5]. Crystal structures of the mammalian SERT and dDAT, show great similarity to the LeuT structure [6 7]. Like most NSSs LeuT has 12 transmembrane domains, as well as a substrate binding site adjacent to two Na^+-binding sites located roughly halfway though the membrane bilayer. [8] LeuT is an amino acid transporter. LeuT undergoes structural conformations in order to transport its solute. Recent studies, based on tmFRET experiments, suggest that LeuT has an outward-open conformation when Na+ is bound, but adapts an outward-closed conformation in the presence of K+, when examining the extracellular side. [2 9]

Förster Resonance Energy Transfer (FRET) is useful for investigating molecular distances. Conventional FRET utilizes the energy transfered from a donor fluorophore, which has been excited by energy from a light source, to an acceptor fluorophore (Figure S1). The FRET efficiency, E, decreases inversely to the sixth power of the distance, R, between the donor and acceptor fluorophore. This is given by the equation $E = R_0^6/(R_0^6 + R^6)$, where R_0 is the critical transfer distance, also known as the Förster distance, and represents the donor-acceptor distance where the efficiency of the energy transfer is 50 %. [10] While FRET is very useful for intermolecular investigations, it is not always suitable for the investigations of intramolecular conformational movements, due to the limited window around

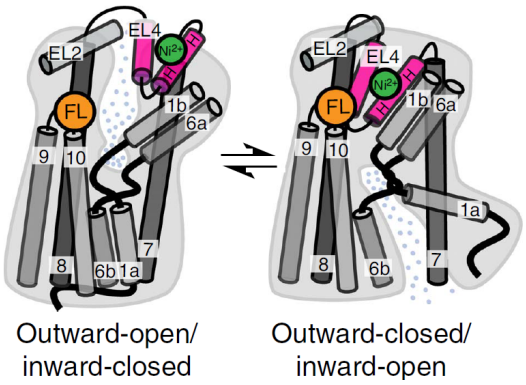

Fig. 1 Cartoon of LeuT with EL4:TM10 tmFRET pair shown in outward open and outward closed states depicting the principle behind changes in tmFRET as a result of conformational changes. The tmFRET pair reports distance-dependent quenching of fluorescence from fluorescien conjugated to an inserted cysteine at the top of TM10 (orange sphere, K398C) by Ni^{2+} coordinated by a His-X3-His motif in EL4 (green sphere, A313H-A317H). Figure adapted from Billesbolle et al [2016][2]

R_0. This is primarily due to the high R_0 values of typical FRET pairs (30-60 Å), as well as the large size of most fluorescent dyes and the dependence upon the orientation of the fluorophores, which all can influence the FRET efficiency.[11]

Transition metal ion FRET (tmFRET), however, has proven to be very useful for investigating intramolecular conformational movements of proteins[12] (Supporting information S1. Theory). Using tmFRET, R_0 values are reduced to about 10 Å.[13] Using tmFRET it is possible to measure the FRET between fluorescien, bound to an inserted cysteine, and a Ni^{2+} ion, which have a R_0 of about 12 Å.[13] The Ni^{2+} ion is bound by a di-histidine motif, which has been introduced into the protein by adding two histidines, spaced one turn apart in an alpha helix[14]. The fluorescien binds to the the cystein by its malamide group, which reacts with the cystein's thiol group. The ability to bind fluorophores to inserted cysteins, is another reason to use LeuT for investigating NSSs. LeuT doesn't naturally have any cysteins, in contrast to many mammalian NSSs, which allows us to insert a cystein strategically, enabling us to utilize it for tmFRET.

To investigate tmFRET in LeuT, we inserted a cystein in K398 at the top of TM10 and a di-histidine site in the helical part of EL4 (Figure 1). The construct K398C-A313H-A317H will from here on be named $LeuT^{tmFRET}$. In order to measure specific binding for FRET it is necessary to remove the signal from the unbound Ni^{2+} ions in the solution. This problem has previously been addressed by also conducting the same measurements on a protein without the di-histidine motif, LeuT mutant K398C ($LeuT^{K398C}$). Thus being able to remove the contribution from the unbound Ni^{2+} by subtracting the $LeuT^{K398C}$ signal from the $LeuT^{tmFRET}$ signal. One study showed that it is possible to entirely remove the effect of Ni^{2+} by adding Zn^{2+} to the solution.[2] As Zn^{2+} is colorless, it should not be able to absorb energy from the relaxation of fluorescien to its ground state. At high enough concentrations the Zn^{2+} ions will competitively inhibit the binding of Ni^{2+} at the di-histidine motif. This should make it possible to use the Zn^{2+} saturated $LeuT^{tmFRET}$ mutant to remove the signal from the unbound nickel, rather than using $LeuT^{K398C}$. In this way it would be possible to completely forgo the $LeuT^{K398C}$ mutant, thereby optimizing the process. The purpose of this paper is to investigate the effect of Zn^{2+} ions binding to the di-histidine motif, aiming to find an alternative to the $LeuT^{K398C}$ mutant for removal of the contribution from the unbound Ni^{2+}.

2 Results and Discussion

2.1 Purification of LeuT

The LeuT mutants were purified twice on two separate occasions. The protein concentration was first examined by absorption at 280 nm. Fractions of 1 mL from the protein elution were examined, and the fractions with the highest protein concentrations were then mixed and aliqouted. The total average protein concentrations for each purification are summarized in table 1. (Further information in Figures S4 and S5)

Table 1 Total protein concentrations from purifications

	1st purification	2nd purification
LeuT(K398C)	0,429 mg/L culture	0,577 mg/L culture
LeuT(tmFRET)	0,105 mg/L culture	0,0496 mg/L culture

The degree of labeling was calcuated to be 64.7 % for $LeuT^{K398C}$ and 74.19% for $LeuT^{tmFRET}$ (Experimental section 3.2) for the first purification.

Protein purity was determined using SDS PAGE (Figure 2). Fraction 1 shows the wash after LeuT was bound the Ni^{2+} resin, where other membrane proteins not bound to the resin can be observed. Fractions A-H show protein elution. Here, the gel shows a clear band at 37 kD, however, we know that LeuT has a molecular weight of about 58 kD[15]. This difference could be due to the common observation of "gel-shifting", when working with membrane proteins. Gel-shifting is a result of the larger amount of hydrophobic domains binding more SDS, therefore making the protein run faster on the gel.[16] A small band at 75 kD can also be observed for $LeuT^{K398C}$. This could perhaps indicate the presence of a LeuT dimer.

Scintillation proximity assay (SPA) was performed in order to investigate amino acid binding of the LeuT mu-

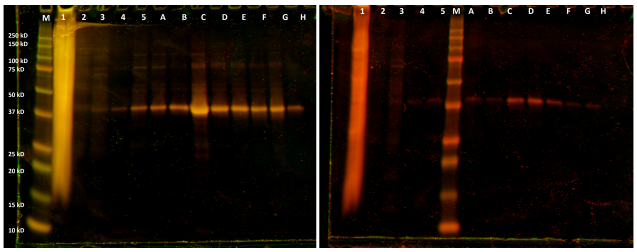

Fig. 2 Determination of LeuT molecular weight using SDS-PAGE from second LeuT purification. Fraction 1 shows the wash after LeuT was bound to the Ni^{2+} resin, fractions 2-5 are from the wash with increasing amounts of imidazole diluted in NSWB (0, 60, 90, 100 mM, respectively), fractions A-H show protein elution with 300 mM imidazole. M indicates the kaleidoscope marker Left) $LeuT^{K398C}$ right) $LeuT^{tmFRET}$

tants compared to wild type LeuT $LeuT^{WT}$ (Supporting information S2. Methods). The histidine tail binds to the YSi-Cu^{2+} HisTag beads. The scintillation liquid in the YSi-Cu^{2+} HisTag beads will then emit photons, when exposed to β-radiation. Radiotracer ^{3}H-leucine was used. The competitive binding between ^{3}H-leucine and amino acids, leucine and alanine, was investigated (Figure 3a and 3b). It was observed that both $LeuT^{K398C}$ and $LeuT^{tmFRET}$ had leucine and alanine binding affinity comparable to $LeuT^{WT}$. Comparing the two, it appears that the binding affinity for leucine is better than for alanine, despite the fact that some studies show that alanine is actually transported more easily by LeuT. This could be due to leucine's binding affinity, being so high that it actually could slow transport [15].

From the SPA data it was possible to determine Half maximal effective concentration EC50 (Table 2). The EC50 values for the LeuT mutants correlate well with $LeuT^{WT}$.

Table 2 EC50 values for LeuT mutants

EC50	$LeuT^{WT}$	$LeuT^{K398C}$	$LeuT^{tmFRET}$
Ala	8.5 μM	15 μM	14 μM
Leu	28 nM	0.1 nM	33 nM

The leucine saturation of the mutants does not diverge much from $LeuT^{WT}$ either (Figure 3c). K_d was determined from the saturation of leucine to be 13 +/- 3 for $LeuT^{WT}$, 7.6 +/- 1.7 for $LeuT^{K398C}$ and for $LeuT^{tmFRET}$ it was determined to 10 +/- 2. Thus, confirming that the mutation of the protein does not appear to have an influence on its affinity for leucine. The sodium dependency on leucine binding was also investigated (Figure 3d). It can be observed that this dependency has been retained in the $LeuT^{K398C}$ and $LeuT^{tmFRET}$ mutants, compared to $LeuT^{WT}$.

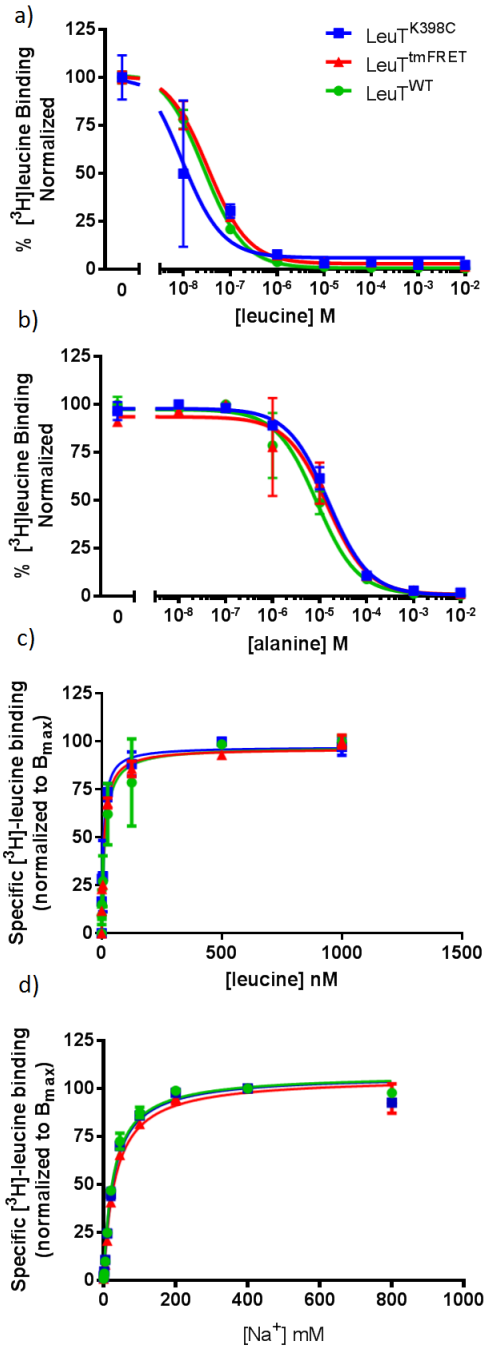

Fig. 3 SPA data shows, LeuT mutants show same saturation and affinity for leucine as wild type LeuT a) $LeuT^{K398C}$ and $LeuT^{tmFRET}$ affinity for leucine correlates with $LeuT^{WT}$ b) $LeuT^{K398C}$ and $LeuT^{tmFRET}$ affinity for alanine correlates with $LeuT^{WT}$ c) Leucine saturation d) Leucine sodium dependency. n=1, performed in duplicates

2.2 tmFRET investigation of LeuT

tmFRET was conducted on $LeuT^{K398C}$ and $LeuT^{tmFRET}$ in 20 mM Tris-HCl pH 8.0, 200 mM ChCl, 100 uM TCEP

and 0.05 % DDM buffer. At increasing concentrations of Ni^{2+}, a saturation of tmFRET signal can be observed (Figure 4). At 10 mM Ni^{2+} there will almost constantly be a Ni^{2+} ion coordinated to the di-histidine site, which will give off the maximum possible tmFRET signal for the protein.

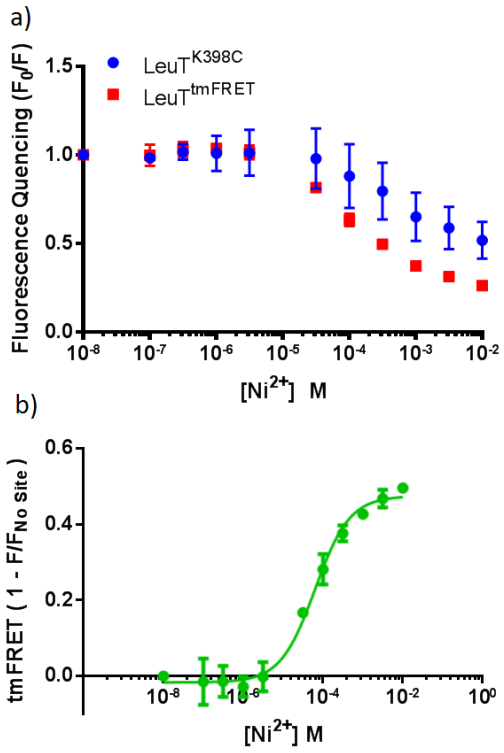

Fig. 4 tmFRET measurements of LeuT a) Fluorescent quenching of $LeuT^{K398C}$ and $LeuT^{tmFRET}$ b) tmFRET of LeuT, where signal from unspecific Ni^{2+} has been removed by subtraction of $LeuT^{K398C}$ signal. n=1, performed in triplicates

2.3 Screening for optimal Zn^{2+} conditions

The buffer used previously for tmFRET on LeuT was a 20 mM Tris-HCl pH 8.0, 200 mM ChCl, 100 uM TCEP and 0.05 % DDM buffer.[2] However, this buffer quickly proved to be unsuitable for solubilizing Zn^{2+} as it precipitated (Figure 5). It was therefor necessary to find a different buffer both suitable for the protein and for solubilizing the Zn^{2+}. As the histidine side chain of LeuT has a pKa of 6.04, the buffer was constricted to pH above about 6.5 in order to keep the histidines from protonating.

In order to determine which component of the buffer was causing the precipitate, 10 mM $ZnCl_2$ was added to four solutions, each missing one component of the original buffer. Each solution was incubated at room temperature for 10 minutes and precipitate investigated. It was found

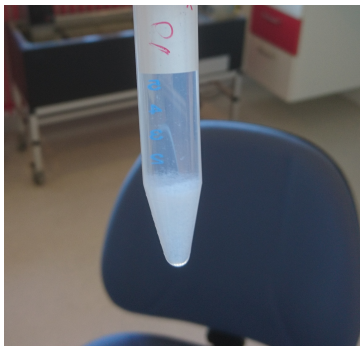

Fig. 5 Zn^{2+} precipitate, 10 mM $ZnCl_2$ in Tris-based fluorescence buffer at pH 8.0

that only the solution without Tris-HCl did not form precipitate. Indeed, it has previously been shown that Zn^{2+} forms complexes with Tris[17]. Other buffer components were investigated to replace Tris. However, the solubility of Zn^{2+} is also highly dependent on pH, therefore the Tris based buffer was also tested at pH 7.5. For all buffers tested, $ZnCl_2$ was added and centrifuged. Surprisingly, the Tris buffer showed no pellets at 10 mM $ZnCl_2$. However, when this buffer was used for tmFRET on the protein with Zn^{2+}, it showed higher FRET signal than other buffers tested (Figure 6c compared to 6a). This indicates that Tris may still be forming invisible complexes with the Zn^{2+} ions, which might prevent Zn^{2+} from binding to the di-histidine motif.

Several other buffers were tested. All buffers were centrifuged with varying concentrations of $ZnCl_2$ for 5 min at 1000 g in order to view pellets from Zn^{2+}. Previous studies suggested buffers from the morpholinic or piperazinic families for the best suitability with Zn^{2+} ions.[18] The results of buffer tests executed are summarized in Table 3. Coincidentally, some of these buffers had buffer ranges at slightly lower pH than the Tris family. It was found that pH was a determining factor in preventing Zn^{2+} precipitation. Additionally, it was found that Tris is an unsuitable buffer component when using Zn^{2+}, as it appears that Zn^{2+} isn't capable of competitively inhibiting Ni^{2+} in the Tris buffer as well as in other buffers, such as MOPS (Figure 6). This is most likely due to complexes formed between Zn^{2+} and Tris. Comparing experiments conducted in the MOPS pH 7.0 and MOPS pH 7.5 buffer, the MOPS pH 7.0 buffer showed slightly lower FRET signals in the presence of Zn^{2+} (Figure 6). This indicates that the MOPS 7.5 buffer could have been continuing to interact with the Zn^{2+} ions, in a manner which disturbed the FRET signal. Therefore, all further experiments were conducted with MOPS pH 7.0 buffer.

Additionally, it was attempted to stabilize Zn^{2+} through the addition of other substrates. Citric acid and ammonium

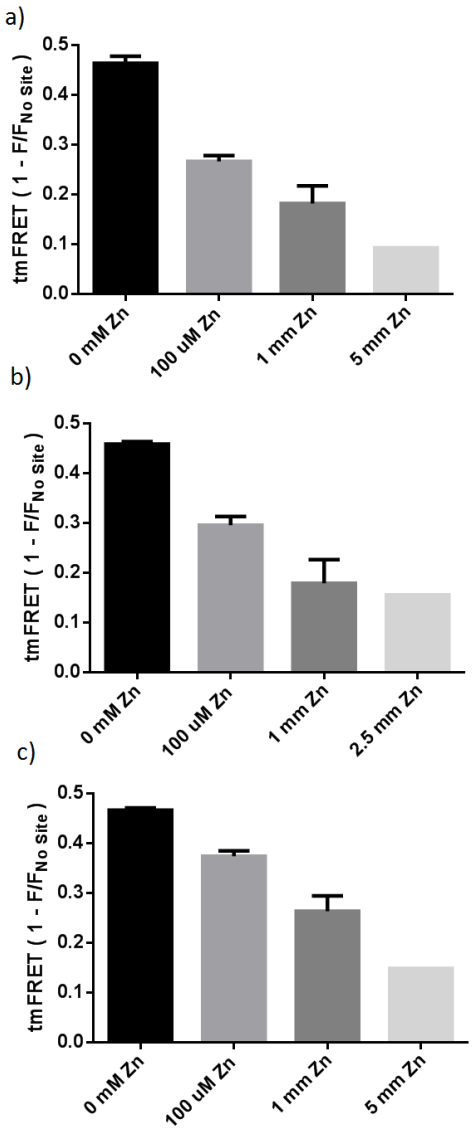

Fig. 6 tmFRET results from LeuT in different buffers at varying Zn^{2+} concentrations with 10 mM Ni^{2+} a) MOPS buffer pH 7.0 b) MOPS buffer pH 7.5 c) Tris buffer pH 7.5, show higher signals in Tris buffer and MOPS pH 7.5 buffer compared to MOPS pH 7.0 buffer. n=1, performed in triplicates

chloride were tested, as these have been found to form complexes with Zn^{2+} [19], which might stabilize the Zn^{2+}, but still allow it to bind the the di-histidine motif. 10 mM of the substrates were added to the chosen buffer and centrifuged with 10 mM Zn^{2+}, in order to view Zn^{2+} pellets. When citric acid was added and the pH was adjusted to 8.0 using KOH, there was no significant difference in pellet size. There was no difference in pellet size observed at the addition of ammonium chloride either, indicating that neither citric acid or ammonium chloride was capable of keeping Zn^{2+} from precipitating.

Table 3 Summary of Zn^{2+} precipitation in different buffers at varying pH

	10 mM Zn^{2+}	2.5 mM Zn^{2+}
Hepes pH 8.0	Pellet	
EPPS pH 8.0	Pellet	
MOPSO pH 7.5	Small pellet	No pellet
MOPS pH 7.5	Small pellet	No pellet
MOPS pH 7.0	Small pellet	No pellet
MOBS pH 8.0	Pellet	Pellet
Tris pH 8.0	Pellet	Pellet
Tris pH 7.5	No pellet	No pellet

2.4 The impact of Zn^{2+} on FRET signal

In order to confirm that the protein still behaved as previously in the MOPS buffer, FRET values for the protein were measured in buffers with different salts; ChCl, NaCl and KCl (Figure 7). In general, the tmFRET values were consistent with values observed in the lab on previous occasions (unpublished).

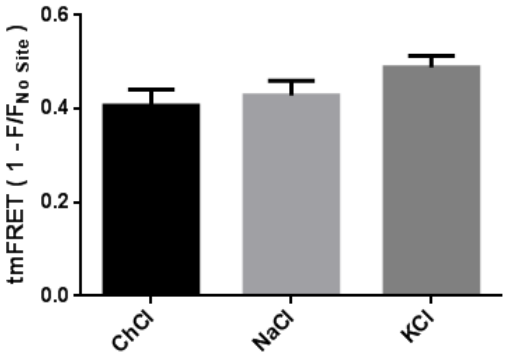

Fig. 7 Effect of different salts on tmFRET signal at 10 mM Ni^{2+}. From the left: ChCl, NaCl and KCl. Slightly heightened tmFRET signal at KCl indicate effect of K^+ on protein conformation. n=1, performed in triplicates

In the KCl buffer we observe a slightly higher FRET value than for the ChCl and NaCl (Figure 7). This could indicate the K^+-effect of conformational change from outward open to inward open conformation, as described in previous studies. [2]

The effect of addition of Zn^{2+} was investigated (Figure 8a). Here it is shown that Zn^{2+} inhibition of Ni^{2+} binding is concentration dependent. At increasing concentrations of Zn^{2+} the saturation point is likewise pushed towards the right, as the Ni^{2+} and Zn^{2+} compete for the di-histidine binding site, also indicating the competitive binding. At 5 mM Zn^{2+} an almost complete inhibition of Ni^{2+} binding was observed. Observing this, it can be deduced that it is possible to use the signal from $LeuT^{tmFRET}$ with 5 mM

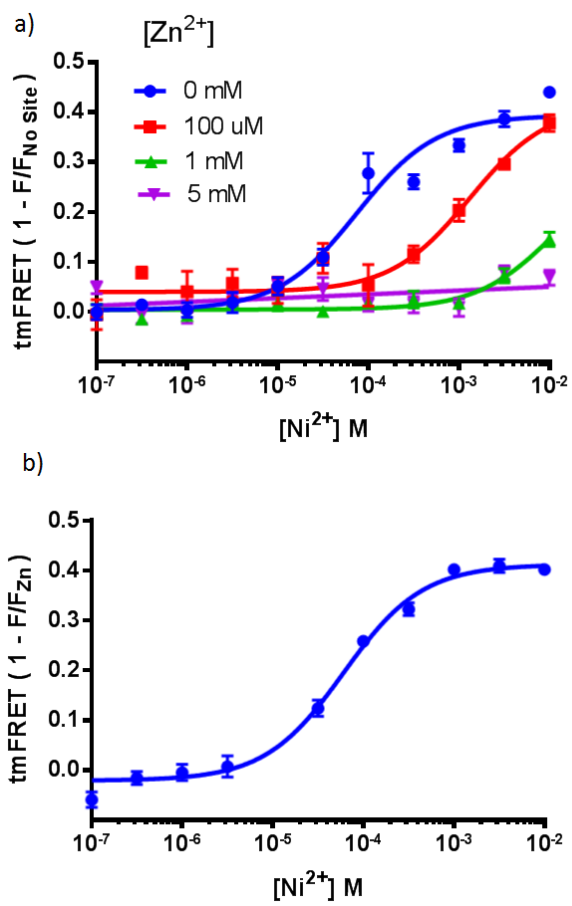

Fig. 8 tmFRET saturation curves at different Zn^{2+} concentrations a) using $LeuT^{K398C}$ to remove contribution from the unbound Ni^{2+} b) using $LeuT^{tmFRET}$ with 5 mM Zn^{2+} to remove contribution from the unbound Ni^{2+}. Data indicates 5 mM of Zn^{2+} can almost completely out-compete Ni^{2+} at the di-histidine motif. n=2, in triplicates

$ZnCl_2$ to remove the contribution from the unbound Ni^{2+} (Figure 8b). This finding should make subtraction of the contribution from $LeuT^{K398C}$ mutant unnecessary, hereby making purification faster and easier, as well as possibly making measurements more consistent as they would be made on the same LeuT mutant.

The concentration dependent inhibition of Ni^{2+} binding to the di-histidine motif can be illustrated as on Figure 9. Here it can be observed how the Zn^{2+} ions increase the fluorescent quenching, by competitively inhibiting Ni^{2+} binding at the di-histidine motif. At 5 mM an almost complete blocking of the signal from the Ni^{2+} bound to the di-histidine motif is observed, hereby rendering the $LeuT^{K398C}$ and $LeuT^{tmFRET}$ essentially identical (Figure 9d).

The effect of Zn^{2+} ions on the intensity was investigted. Despite the fluorescence quenching signals of $LeuT^{K398C}$

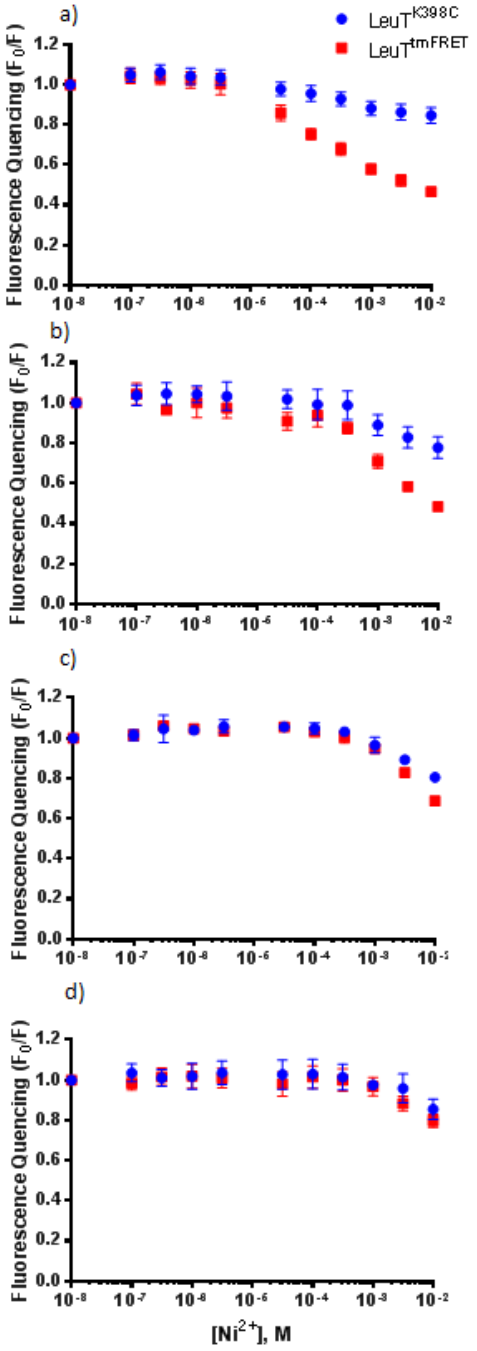

Fig. 9 Fluorescent quenching at increasing concentrations of Zn^{2+} a) 0 mM Zn^{2+} b) 100 uM Zn^{2+} c) 1 mM Zn^{2+} d) 5 mM Zn^{2+} shows increasing quenching of the $LeuT^{tmFRET}$ mutant. n=2, in triplicates

and $LeuT^{tmFRET}$ appearing identical (Figure 9), an effect of Zn^{2+} on the protein was observed (Figure 10). The intensity was measured on the $LeuT^{K398C}$ mutant with increasing concentrations of Zn^{2+} (Figure 10a). Here, the presence of Zn^{2+} surprisingly increases the intensity. This

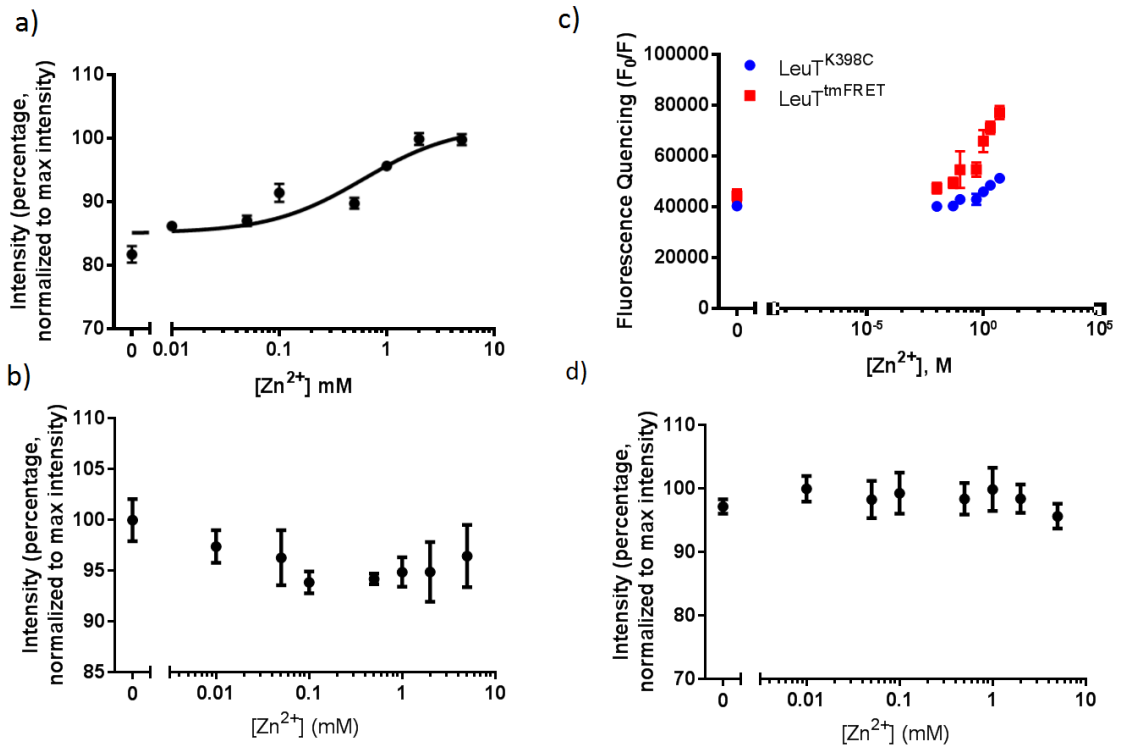

Fig. 10 Zn^{2+} titration curves a) $LeuT^{K398C}$ shows increasing intensity at increasing concentrations of Zn^{2+}, when incubated for one hour before measurements b) $LeuT^{K398C}$ shows no difference in intensity at increasing concentrations of Zn^{2+}, when measured immediately after addition of Zn^{2+} c) Both $LeuT^{K398C}$ and $LeuT^{tmFRET}$ with 10 mM Ni^{2+} show increasing intensity at increasing concentrations of Zn^{2+} d) Zn^{2+} titration curve with fluorescien, but without protein shows no significant difference at increasing concentrations of Zn^{2+}. a and c n=2, in triplicates. b and d n=1, in triplicates

increase in intensity is not observed if measured immediately after addition of Zn^{2+} (Figure 10b), indicating that the sample may need time to potentially form complexes or react with LeuT.

The titration of Zn^{2+} in both the $LeuT^{K398C}$ and $LeuT^{tmFRET}$ mutant in the presence of 10 mM Ni^{2+} was investigated (Figure 10c). Here we also see an increase of intensity in both the $LeuT^{K398C}$ and $LeuT^{tmFRET}$ mutant in the presence of Zn^{2+}. In order to investigate if this property was a function of Zn^{2+} and the protein itself or of Zn^{2+} and the fluorescien directly, Zn^{2+} was titrated into buffer containing free fluorescien, not coupled to LeuT (Figure 10d). This indicates that the Zn^{2+} concentration does not have an influence on the signal, when the protein is not present. This indicates that the increased intensity must be due to an effect Zn^{2+} has on LeuT, which in turn, influences the fluorescien intensity.

If this increase in intensity from Zn^{2+} comes from complexes or an effect on the protein, it poses a problem in using a Zn^{2+} inhibited $LeuT^{tmFRET}$ to subtract the signal from the unbound Ni^{2+}, as it won't be completely accurate at high concentrations. No great difference in intensity as a function of Zn^{2+} concentration is observed on the $LeuT^{K398C}$ signal till about 2 mM (Figure 10c). This could allow the usage of Zn^{2+} inhibited $LeuT^{tmFRET}$ to subtract the signal from the unbound Ni^{2+}, only using a lower concentration of Zn^{2+}. It appears that a concentration of 1 mM Zn^{2+}, does not change the intensity and thus could be used (Figure 10c). This would not allow as high a concentration of Ni^{2+}, as the lower Zn^{2+} concentration would not be able to competitively inhibit it, thus removing the signal. It can be deduced that a concentration of 1 mM Ni^{2+}, should be able to be competitively inhibited by 1 mM Zn^{2+} (Figure 8a). While not being able to do a full saturation curve with only 1 mM Ni^{2+}, it still allows for a relatively good indication of comparative FRET and conformation of the LeuT protein. Even with these limitations, this method has the advantage of being able to do measurements on the same protein.

This allows measurements to be more consistent, as they will no longer be a comparison between two different mutants of LeuT. This will make it possible to forgo the purification of $LeuT^{K398C}$, thereby optimizing the purification process. However, this increased intensity could also be due to the inner filter effect. The inner filter effect has previously been observed in the lab from Ni^{2+}. If this is

the case, using Zn^{2+} inhibited LeuT^{tmFRET} may actually offer a better solution, as it would not only only allow us to conduct the measurements on the same protein, but it would also allow for the subtraction of the inner filter effect. To determine if these findings are significant, further experiments would have to be conducted and statistical tests would have to be done.

3 Experimental section

3.1 Purification of LeuT

Unless otherwise stated, all chemicals used were purchased from Sigma-Aldrich. Two mutants of LeuT were purified; A313H-A317H-K398C (LeuT^{tmFRET}) and K398C (LeuT^{K398C}) - one with and without the dihistidine motif.

For the purification the following buffers were used: Lysis buffer (LYB), Sucrose buffer (SUB), Solubilization buffer (SOB) and No Sodium Wash Buffer (NSWB).

Lysis buffer: 100 mM KCl, 50 mM Tris-Hcl (pH 8.0)

Sucrose buffer: 100 mM KCl, 50 mM Tris-HCl (pH 8.0), 1mM EDTA, 0.5 M sucrose

Solubilization buffer: 50 mM Tris-HCl (pH 8.0), 30 % glycerol, 300 mM KCl, 5 mM MgCl2, 1 mM TCEP

No Sodium Wash Buffer: 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 100 uM TCEP, 20 % glycerol, 0.05 % n-dodecyl-β-D-maltosepyranoside (DDM) (Anatrace)

One aliquot freestock of the E. Coli Stock strain C41, transformed with the desired LeuT construct was added to Lysogeny Broth containing 75 ug/ml ampicilin and grown overnight at 37C with 180 rpm shaking.

50 ml inoculation culture was added to 1000 mL LB containing ug/ml ampicilin and grown for 2.5 hours at 37C. Protein expression was induced at OD600 0.5-0.6 by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was then incubated at 20C with 180 rpm shaking overnight.

The cells were pelletzied for 10 min at 6000 rpm 4 C in Sorvall LYNX 6000 Superspeed centrifuge (Thermo Fisher Scientic Inc.). The pellets were then resuspended in lysis buffer and 10 ml sucrose buffer, 200 uL of 0.2 M phenylmethylsolfonyl fluoride (PMSF) , 200 uL protease inhibitor (PI) was added to each pellet. The cells were ruptured through double passage through high-pressure cell disrupter (Constant Systems Ltd) at 2.30 bar and flushed with supplemented solubilization buffer (SOB). The solution was centrifuged for 15 min at 1000 g at 4C in Sigma 6K15 centrifuge (SciQuip Ltd) and subsequently the supernatant was ultracentrifuged in OptimaTM L-80 XP Ultracentrifuge (Beckman Coulter, Fullerton) for 2 hours at 125171 g at 4C. Hereafter, the pellet was resuspended in supplemented Solubilization buffer. Membrane fractions were then stored at -80C.

n-dodecyl-β-D-maltosepyranoside (DDM) from Anatrace was then added to a final concentration of 1 percent in order to solubilize LeuT, and then incubated for 1 hour at 4C under slow rotation. LeuT was bound to ProBond Ni-IDA resin (Life Technologies), afterwards it was incubated in No Sodium Wash Buffer (NSWB). The membrane fraction was pelletized by centrifugation at 38.000g for 30 min at 4C by Sorvall LYNX 6000 Superspeed centrifuge (Thermo Fisher Scientic Inc.). Fluorescein-5-maleimide (FL) was added dropwise to a final concentration of 200 uM for fluorescent conjugation.The protein-bound resin was then incubated for 16 hours under slow rotation at 4C, away from light.

Protein bound resin was centrifuged by Sorvall LYNX 6000 Superspeed centrifuge (Thermo Fisher Scientic Inc.) at 1.000 rpm for 3 min at 4C with NSWB and the pellets were then washed with NSWB containing imidazole in order to remove unconjugated fluorescien. Protein elution was done with 300 mM imidazole. SDS PAGE analysis was run in order to quantify protein amount in obtained fractions. Furthermore, protein content was determined at absorbance at 280 nm with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientic Inc.), correcting for the protein being fluorescently labeled. Aliquotes of 100 uL were then made and stored at -80C.

3.2 Calculation of protein concentration and degree of labeling

The total protein concentration of the collected fractions was determined using Lambert-Beers law: $C = \frac{A}{\varepsilon * l}$, where A is the absorbance, ε is the molar absorptions coefficient and l is the length of the light path. Using ε for LeuT 113300 $\frac{L}{mol*cm}$ and ε for fluorescien 80000 $\frac{L}{mol*cm}$. Protein concentration for LeuT^{K398C} was determined to 6.8 uM (1st purification) and 9.9 uM (2nd purification). For LeuT^{tmFRET} the protein concentration was determined to 3.1 uM (1st purification) and 1.5 uM (2nd purification). For fluorescein the concentration was determined to 4.4 uM (1st) and 5.3 uM (2nd) for the LeuT^{K398C} mutant. For LeuT^{tmFRET} the concentration was determined to 2.3 uM (1st) and 1.6 uM (2nd), for fluorescien. The absorbance was determined by absorption at 480 nm for fluorescien. From these protein concentrations the degree of labeling was calculated as [LeuT$^{K398C}{}_{FL}$]/[LeuT^{K398C}] and [LeuT$^{tmFRET}{}_{FL}$]/[LeuT^{tmFRET}]

3.3 tmFRET

Samples were prepared for tmFRET by the addition of 0.0005 mg/mL protein to the fluorescence buffer (20 mM MOPS-KOH buffer pH 7.0 , 200 mM ChCl, 100 uM TCEP and 0.05 % DDM).

For the saturation curves, $ZnCl_2$ was added to each sample at 100uM, 1 mM and 5 mM, respectively. The sample was mixed well and 330 uL was transferred to each eppendorf tube. Samples were then incubated for one hour at room temperature, shielded from light. After incubation, 3.3 uL from the following dilution series was was added to each tube: $1*10^{-5}$, $3.16*10^{-5}$, $1*10^{-4}$ M, $3.16*10^{-4}$ M, $1*10^{-3}$ M, $3.16*10^{-3}$ M, $1*10^{-2}$ M, $3.16*10^{-2}$ M, $1*10^{-1}$ M, $3.16*10^{-1}$ M, 1 M. The samples were immediately measured on the FluoroMax 4 spectrofluorometer (Horiba Scientic Ltd.).

For the Zn^{2+} titration curves a similar approach was used. Zn^{2+} was added to the sample in increasing concentrations, 0 M, 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM and 5 mM, respectively. The sample was mixed well and 330 uL of the mixture was transfered to each eppendorf tube. The samples were then incubated at room temperature, shielded from light for one hour. For the experiment shown on Figure 10c, 10 mM Ni^{2+} was then added to each sample. All samples were then immediately measured on the FluoroMax 4 spectrofluorometer (Horiba Scientic Ltd.).

Settings for the FluoroMax 4 spectoflurometer: Excitation slit width: 3.5, emission slit width: 3.5, wavelength excitation: 496 nm, wavelength emission 519 nm. All measurements were conducted in triplicates.

4 Conclusions

In this study, tmFRET was used for investigating the competitive binding of Zn^{2+} and Ni^{2+} at the di-histidine site of LeuT mutant A313H-A317H-K398C ($LeuT^{tmFRET}$). Suitable buffers were investigated, and it was found that Tris based buffers were unsuitable, while MOPS based buffers showed great promise. It was furthermore found that the pH of the buffer needed to be below 7.5 in order for the setup to function. It was found that Zn^{2+} was capable of inhibiting Ni^{2+} binding, indicating that a Zn^{2+} saturated $LeuT^{tmFRET}$ could be used to remove the signal from unbound Ni^{2+}. However, indications that Zn^{2+} in concentrations above 1 mM increased the fluorescence intensity was also found. The described technique, however, can still be utilized at Zn^{2+} concentrations below 1 mM. Doing so limits the concentration of Ni^{2+} as well, but it does allow for investigation of the conformational changes of the protein. The increase in fluorescence intensity could, however, also be due to inner filter effect. This finding will allow tmFRET measurements to be more consistent as they can be performed on the same LeuT mutant $LeuT^{tmFRET}$, as well as optimize the purification process as the $LeuT^{K398C}$ mutant would be rendered obsolete.

5 Acknowledgements

I would like to express my gratitude towards my supervisor Claus Juul Løland, associate professor, Institute for Neuroscience and pharmacology at Copenhagen University for great guidance and constructive feedback throughout the last few months.

I would like to thank my internal supervisor Nikos Hatzakis, associate professor, Department of Chemisty at Copenhagen University for making it possible for me to write my thesis at Center for Neuroscience.

I would like to thank Jonas S. Mortensen, PhD at Center for Neuroscience at Copenhagen University, for the great help he has provided, introducing me to the lab and guiding me throughout the entire project.

I would also like to thank Solveig G. Schmidt for always being helpful and available for both discussions and help in the laboratory.

I would also like to thank lab technician Lone Rosenquist, as well as the entire Neuropharm and Genetics Lab for help during the day to day lab experiments.

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