Sizing Analysis of the Ubiquitin / E1-E2-E3 Complex using switchSENSE® technology

Keywords: ubiquitin, E1, E2, E3, sizing, charge/discharge reaction

During the posttranslational process of ubiquitination, the small regulatory protein ubiquitin (Ub) is covalently attached to a lysine residue of a substrate protein in an enzymatic cascade to label target substrates for proteasomal degradation[1]. Alterations in the ubiquitination pathway have been implicated in several diseases, such as in neurodegenerative diseases (e.g. Parkinson's diseases) as well as in a variety of different types of cancers[2,3].

Here, we report a novel approach to study the charge reaction of ubiquitin to the ubiquitin-conjugating enzyme (E2) mediated by the ubiquitin-activating enzyme (E1), as well as the subsequent transfer of ubiquitin to a lysine in a ubiquitin ligase (E3)-dependent discharge reaction using switchSENSE® size analysis.

Methods and Results

A switchSENSE® Multi-Purpose Chip with 48 bp DNA nanolevers labeled with a red fluorophore (MPC2-48-2-R1) was used for the experiments. Measurements were performed with a single color DRX instrument. Ubiquitin was conjugated to DNA (cNL-B48), which was complementary to the pre-immobilized DNA nanolever, via amine coupling and used to functionalize the surface. Subsequently, E2 (UbcH5B) was charged with ubiquitin by an E1 (Uba1) in a Mg^{2+}-ATP- dependent reaction to form a E2~Ub complex in which ~ denotes a thioester between the C-terminal tail of Ub and the catalytic Cys of the E2, and the difference in the hydrodynamic (Stokes) diameter (D_H) was investigated in a switchSENSE protein sizing experiment[4]. Subsequently, Ub was transferred from the E2~Ub complex to free lysine in an E3 (RNF38)-driven discharge reaction.

Step 1 – Preparation of DNA-ubiquitin conjugates

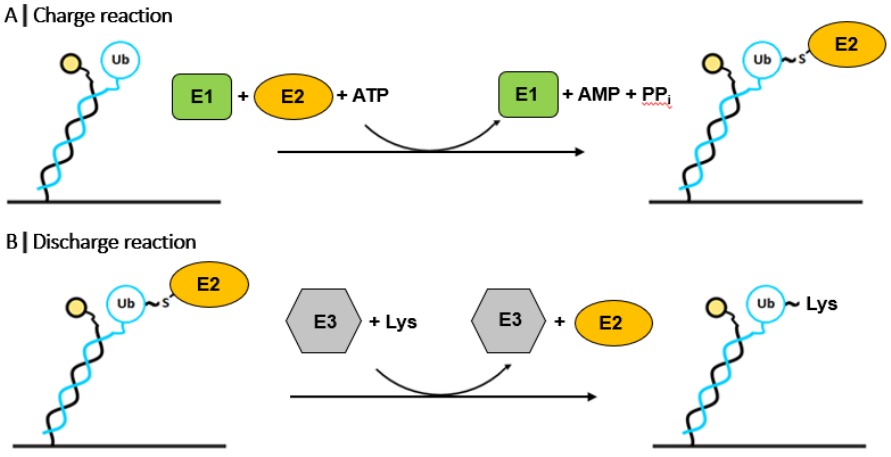


Figure 1 I Multi-step assay on an electro-switchable sensor surface: Ubiquitin E1-E2-E3 interaction. (**A**) Charge reaction: Mg^{2+}-ATP dependent conjugation by E1 of ubiquitin to E2 to form E2~Ub (**B**) Discharge reaction: E3 mediated transfer of ubiquitin to free L-lysine

To functionalize the sensor surface with ubiquitin, we prepared conjugates between Ub and single stranded 48mer DNA (cNL-B48) using the amine coupling kit CK-NH2-1-B48. Subsequently, the Ub-DNA conjugate was hybridized to its complementary DNA on a Multi-Purpose Chip (MPC2-48-2-R1), yielding double-stranded DNA nanolevers. Hybridizations of the DNA-Ub conjugates were performed at 500 nM in TE40 buffer (10 mM Tris-HCl, pH 7.4, 40 mM NaCl, 0.05 % Tween20, 50 µM EDTA, 50 µM EGTA). At this given conjugate concentration, one conjugation reaction yielded a total number of 40 chip functionalizations. By increasing the hybridization time, conjugate concentrations as low as 100 nM can be used, therefore increasing the number of possible chip functionalizations to 200. Hybridizations were followed in real-time using the standard routine of the DRX instrument.

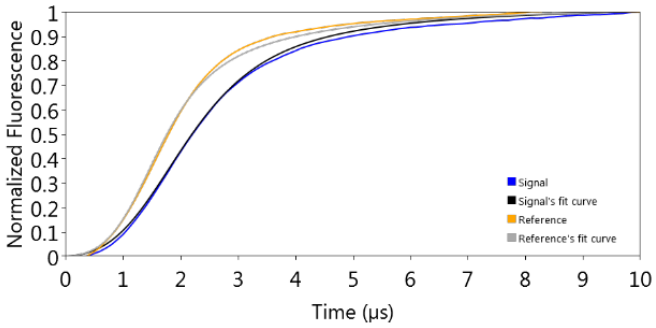
Step 2 – Sizing of ubiquitin

Ub-DNA conjugate (cNL-B48-Ub) was immobilized on the sensor surface and the protein's switching dynamics were recorded. By comparison to the switching dynamics of unmodified double-stranded DNA (cNL-B48), the switchANALYSIS software determined the hydrodynamic diameter (D_H) of ubiquitin. Figure 2 shows time-resolved data of the upward switching motion: the DNA-Ub conjugate features a higher hydrodynamic friction and lags behind the faster unmodified DNA nanolever. The **switch**SENSE® derived hydrodynamic diameter (D_H^{avg}) of 3.8 nm (Fig. 2) and the calculated diameter from the crystal structure (D_H^{pdb}) (pdb: 1UBQ) of 3.4 nm are in good agreement.

Step 3 – E1-mediated charging reaction of Ub to E2 to form E2~Ub

Next, we investigated the covalent attachment of ubiquitin of E2 in a E1-Mg^{2+}-ATP manner. First, conjugate was immobilized on the surface.

DNA-Ub conjugate was immobilized on the surface. In a subsequent step a constant concentration of E1 (200 nM) together with different concentrations of E2 (0 µM, 0.625 µM, 1.25 µM, 2.5 µM) diluted in running buffer (58.5 mM Tris-HCl, pH 7.6, 18.5 mM NaCl, 5 mM ATP, 5 mM $MgCl_2$, 0.01 % Tween20, 9 µM EDTA, 9 µM EGTA,



D_h^{avg} = 3.83 ± 0.10 nm (n=3)

Figure 2 I Determination of the hydrodynamic diameter D_H of ubiquitin from the upward switching fluorescence response for unconjugated reference DNA (orange) and the protein-DNA conjugate (blue).

9 µM TCEP, 1 mg/ml BSA) was injected into the flow channel at a flow rate of 30 µl/min for 15 min to allow for the E1-dependent enzymatic reaction between ubiquitin and E2. After a dissociation step to remove excess enzyme and substrate (flow rate of 100 µl/min for 20 min), we determined the hydrodynamic diameters. Before each E1/E2 injection, the sensor surface was functionalized with fresh Ub conjugate. Upon attachment of E2 to Ub, the size of the complex increased from D_H = 3.8 nm for bare Ub to D_H = 5.5 – 6.0 nm for the Ub~E2 complex, cf. Figure 3. We found that the lowest applied E2 concentration yielded a pronounced increase in D_H. Nevertheless, the hydrodynamic diameter increased slightly when using higher concentrations of E2, indicating that full saturation had not yet been reached at low concentrations of E2. As a negative control, we omitted the E1 enzyme from the reaction in one set of experiments; in this case the hydrodynamic diameter remained constant, confirming that the charge reaction is indeed enzyme-dependent.

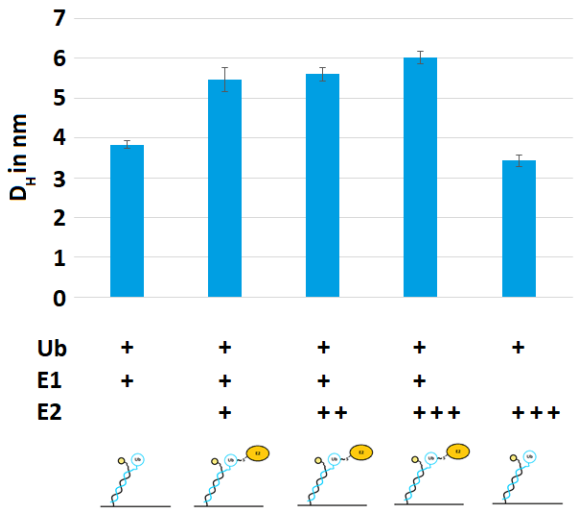


Figure 3 I E1 mediated formation of E2~Ub complexes during the charge reaction. Bars show hydrodynamic diameters determined by switchSENSE sizing measurements for different concentrations of E2 substrates (0 µM, 0.625 µM, 1.25 µM, 2.5 µM) as well as a negative control (without E1).

Step 4 – Influence of L-lysine on the charge reaction

To study the transfer of ubiquitin to a substrate by the E1-E2-E3 machinery, the sensor was functionalized with ubiquitin-DNA conjugate nanolevers as before. In a sandwich assay, first E1 (200 nM) and E2 (2.5 µM) were added to form the E2~Ub complex on the nanolever (15 min at a flow rate of 30 µl/min). Then we used a 15 min dissociation step at a flow rate of 100 µl/min to remove excess enzyme and substrate. We determined the hydrodynamic diameter after each step, which were all conducted in 'charge buffer' with or without 20 mM L-lysine in order (i) to provide a substrate for the subsequent transfer reaction in step 5, and (ii) to determine its influence on the charge reaction. The sizing data show that the presence of L-lysine during the charge reaction prevents the formation of the E2~Ub complex (D_H = 3.9 nm), i.e. L-lysine acts as a competitor. However, in the absence of L-lysine, E2 can be attached to ubiquitin successfully, which is evidenced by the larger diameter (D_H = 5.4 nm) (Fig. 4).

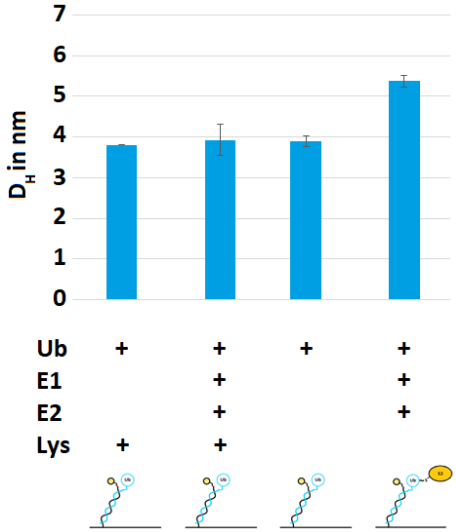


Figure 4 I Inhibition of charge reaction by L-lysine as monitored by measuring D_H.

Step 5 – Discharge reaction: Transfer of Ub from E2~Ub to L-lysine

The sensor was functionalized with ubiquitin-DNA conjugate as before. For the charge reaction samples were diluted in charge buffer without L-lysine (= running buffer) and injected for 15 min at 30 µl/min. For the subsequent discharge step, the sensor was incubated with 2.5 µM E3-ligase, (20 min at 30 µl/min) followed by a dissociation/wash step (15 min at 100 µl/min) in discharge buffer with L-lysine (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 150 mM L-lysine, 1 mg/ml BSA). The hydrodynamic diameter D_H was determined after each step of the reaction. The sizing data clearly evidence a successful discharge reaction, i.e. the removal of E2 from the ubiquitinated nanolevers in the presence of E3 ligase and L-lysine as shown by a decrease of D_H. This discharge reaction is dependent on both the presence of L-lysine as well as a catalyzing enzyme (E3) (Fig. 5).

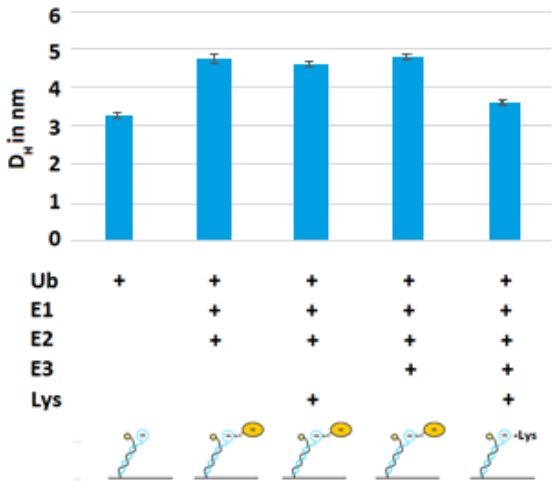


Figure 5 I Transfer of ubiquitin to substrate analogue L-lysine in the presence of E3 during the discharge reaction

Conclusions

The **switch**SENSE® technology allows monitoring of an essential pathway in proteostasis, namely the interaction between ubiquitin, the E1-E2-E3 machinery and a substrate molecule.

- Ubiquitin could be conjugated to DNA nanolevers and hybridized on the **switch**SENSE® sensor. Its hydrodynamic diameter (D_H^{avg}) could be determined and is in very good agreement with crystal structure data.
- The ubiquitin-activating enzyme (E1) dependent covalent linkage of ubiquitin to ubiquitin-conjugating enzyme (E2) to form the E2~Ub as well as the subsequent transfer of ubiquitin to L-lysine in the presence of ubiquitin ligase (E3) can be followed by changes of the hydrodynamic diameter.

Advantages compared to other experimental approaches

- Fast detection. One screening run including charge and discharge reaction takes less than one hour.
- Automation. All DRX instruments operate fully automated, allowing to test many different samples and reaction conditions with only little hands-on time.
- No hazards. There is no need for radioactive isotope labelling.

References

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November 2018

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