UbiQ
targeting the ubiquitin system

Structure determination

version	V1, July-2016
applicable for	UbiQ Triple E Probes, Activity-based probes for E1-E2-E3 enzymes
reference	Mulder, M. P. C. et al. a cascading activity-based probe sequentially targets E1–E2–E3 ubiquitin enzymes. *Nature chemical biology.* **12,** 523–530 (2016). doi:10.1038/nchembio.2084.

protocol

Preparation of thioether-linked [^{15}N]UBE2N–Ub-Dha and UBE2D3–Ub-Dha.

1. incubated at room temperature for 16 h:
    - 25 µM UBE1, 250 µM E2 ([^{15}N]UBE2N or UBE2D3),
    - 1 mM Ub-Dha, 5 mM ATP, and
    - 10 mM $MgCl_2$
2. at the end the remaining thioester linkages were reduced by the addition of 10 mM DTT
3. [^{15}N]UBE2N–Ub-Dha adduct was purified to ~95% homogeneity by size-exclusion chromatography on a Superdex 75 column (GE Healthcare) equilibrated in:
    - 25 mM sodium phosphate (pH 7.0)
    - 150 mM NaCl

UBE2D3–Ub-Dha adduct was subsequently purified to >98% homogeneity by:

1. repeated cation exchange chromatography on a 6 mL resource S column (GE Healthcare) with a
    a. 50–500 mM NaCl gradient
    b. in 50 mM Tris (pH 8.5) buffer
2. a size-exclusion chromatography step on a Superdex 75 column (GE Healthcare) equilibrated in 25 mM HEPES, 50 mM NaCl, pH 7.

NMR spectroscopy with [^{15}N]UBE2N–Ub-Dha.

1. ^{1}H,^{15}N BEST-TROSY experiments on
    a. 200 µM [^{15}N]UBE2N or
    b. [^{15}N]UBE2N–Ub-Dha
2. were acquired with optimized pulse sequences on a Bruker Avance2+ 700MHz spectrometer equipped with a cryogenic triple resonance TCI probe.
3. data sets were processed using Topspin (Bruker)
4. visualized with NMRViewJ (One Moon Scientific)
5. Chemical shift perturbations were calculated using the equation $Ää_j = [(Ää_j{}^{15}N/5)^2 + (Ää_j{}^{1}H)^2]^{1/2}$
6. perturbation values greater than 0.05 p.p.m. were mapped onto the UBE2N crystal structure (PDB 1J7D), such that the analysis was directly comparable to previous work on the oxyester-linked UBE2N–O~Ub conjugate

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Crystallization, data collection and structure determination for UBE2D3– Ub-Dha.

Thioether-linked UBE2D3–Ub-Dha adduct was crystallized following conditions published for the oxyester-linked form.

1. purified conjugate was concentrated to 0.9 mM (23.5 mg/mL)
2. screened against conditions surrounding the published 200 mM tripotassium citrate, 20% PEG 3350 condition in 200 nL sitting drops at a 1:1 protein/reservoir ratio.

3. crystals were cryoprotected in LV CryoOil (MiTeGen)
4. vitrified prior to data collection at Diamond Light Source beamline I04-1
5. data were collected at 100K with a wavelength of 0.92 Å
6. diffraction images were processed using XDS and scaled using AIMLESS

7. Isolated UBE2D3 and Ub molecules from the oxyester UBE2D3–O~Ub structure (PDB 3UGB) were used as search models for molecular replacement using PHASER.
8. Iterative model building and refinement were performed using COOT and PHENIX, respectively.
9. the thioether linkage could be built unambiguously into the electron density.
10. the final model contained 96.9% in favored, 2.7% in allowed, and 0.4% in outlier Ramachandran space
11. structural figures were generated using PyMOL (http://www.pymol.org).

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