

The insulin degrading enzyme activates ubiquitin and promotes the formation of K48 and K63 diubiquitin.

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Supplementary Info.

Materials and Methods

Chemicals: Ub from bovine red cells was obtained from Sigma Aldrich and purified by extensive dialysis against pure water for 24 h at 48 °C, as reported elsewhere. Protein concentration in pure water was routinely measured by UV ($\epsilon_{280} = 1280 \text{ M}^{-1} \text{ cm}^{-1}$). Dithiothreitol (DTT) was purchased from Fluka. In order to verify the reproducibility of the experiments on different enzyme batches, IDE was purchased either from Calbiochem or GIOTTO Biotech s.r.l. (Italy). Human recombinant Ub activating Enzyme (UBA1), UbcH13-UeV1 and E2-25K (E2) were purchased from Boston Biochem. Ammonium molybdate heptahydrate, sodium pyrophosphate were obtained from Aldrich and used without further purification. The solution containing 400 mM EDTA and 1,4 M NaOH was prepared and used to terminate the enzymatic reactions. For SDS PAGE NuPAGE® Novex® Bis-Tris gels and 2-(N-morpholin) ethanesulfonic acid (MES) buffer were obtained from Invitrogen. For Western blotting, mouse anti mono- and poly-ubiquitinated conjugates, mAb (FK2) and anti-rabbit poly-ubiquitinated conjugates, mAb (D4D6) were obtained from ENZO Life Science and Cell Signalling, respectively. The ubiquitylation kit was purchased by ABCAM (139472). All solutions were prepared with ultrapure Milli Q water.

Lys48 and Lys63 self-polyubiquitination reactions in tube tests. Lys48-linked polyubiquitination reactions were performed at pH 7.4 ($T=25^{\circ}\text{C}$) in small volumes (40 μL) of ligation buffer (50 mM MOPS, 5 mM MgCl_2 , 30 μM DTT and 2 mM ATP) containing Ub (10 μM), UBA1 (500 nM) and E2-25K (1 μM). The same procedure was followed for Lys63-linked polyubiquitination reactions, using the ligation buffer containing Ub (5 μM), UBA1 (100 nM), UbcH13-UeV1 enzymes (500 nM). Different concentrations of IDE were tested in the mixtures instead of UBA1 and E2 enzymes. The reactions were quenched after 3 hours incubation and size-fractionated by SDS-polyacrylamide gel electrophoresis. They were then electro-transferred onto a nitrocellulose membrane (GE Healthcare, Lifescience). The membranes were blocked with Odyssey blocking buffer for 1 hour and then incubated overnight at 4°C with K48-linkage specific polyubiquitin antibody and K-63-linkage specific polyubiquitin antibody, respectively. The membrane was washed thrice for 5 minutes with PBS-T (PBS, 0.05% Tween 20) and then incubated with IRDye 800-labeled secondary antibody (1:12000) from Molecular Probes (Eugene, OR) for 30 minutes. Visualization of membrane was done using the LI-COR Odyssey IR Imaging System (LI-COR Biosciences, Lincoln,

Ubiquitin activation. The monitoring of formation of activated ester covalent linkage between Ub2 and E1 using the ubiquitylation kit ABCAM (139472). The Ub adenylate formation was obtained using reaction mixtures containing 2.5 μM E1 or IDE, 50 μM Ub, 2mM ATP in 10 mM MgCl_2 , 50 mM TRIS, pH 7.5. The reaction mix were carried out under stirring at 37°C for 30 min before an aliquot of the sample loading buffer was added to quench the reaction. The samples were then analyzed by SDS-PAGE under reducing conditions, transferred onto a nitrocellulose membrane (GE Healthcare, Lifescience), probed with mouse anti mono- and poly-ubiquitinated conjugates mAb [clone FK2] (1:5000), overnight at 4°C . After three washes for 5 minutes with PBS-T (PBS, 0.05% Tween 20), the membrane was incubated with IRDye 800-labeled secondary antibody (1:12000) from Molecular Probes (Eugene, OR) for 30 minutes. Visualization of bands was performed on a Li-Cor Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE).

Spectrophotometric determination of $\text{P}_2\text{O}_7^{2-}$ anion in ubiquitin activation tube tests. The ubiquitin chain elongation reactions were carried out for one hour as described in the previous paragraph. The reaction mixtures (10 μL) were collected every five minutes in a 96 well plate and analysed by a Varioskan plate reader. In each well, 10 μL of a 400 mM EDTA solution were used to quench all reactions. Then, solutions were incubated for 10 min with 200 μL of 3 mM ammonium molybdate in 0,6 M HCl (60% AcN/W). The colorimetric reaction was started by adding 80 μL of a solution of

ascorbic acid (500 mM) in 2M HCl (60% AcN/W). After 10 min the optical absorbance was measured at 790 nm. Calibration curves for accurate pyrophosphate measurements were obtained by using standard solutions containing different pyrophosphate concentrations (0-70 μ M).

NMR spectroscopy. NMR experiments were acquired at 298 K by a Varian Unity INOVA 500 MHz spectrometer. The NMR samples were prepared as follows. For chemical shift mapping studies of human-Ub in presence of unlabeled IDE, ^{15}N - ^{13}C labeled Ub (purchased at Giotto Biotech srl, Sesto Fiorentino Italy) was dissolved in 500 μ L at 100 μ M concentration in 50 mM phosphate buffer pH 7 and 10% D_2O (final volume of the sample 550 μ L). Unlabeled IDE was added as increments of known amounts dissolved in the same buffer, obtaining a final concentration of 10 μ M (Ub:IDE ratio of 1:10). After each addition of IDE solution, the volume of the sample has been concentrated back to 550 μ L using Centricons. ^1H , ^{13}C , and ^{15}N chemical shifts were calibrated indirectly by using external references. All NMR data were processed with the software VNMRJ 1.1.D (Varian Inc.) and analyzed using the CARA (Computer Aided Resonance Assignment) software (downloaded from cara.nmr.ch).¹ The intensity variations of the amide cross-peaks in the presence and absence of IDE were calculated using the following equation: $\Delta I = (I - I_0)/I_0$, where I_0 and I are the amide cross-peak intensities in absence and in presence of IDE, respectively.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) measurements were carried out on a SensiQ Pioneer instrument, and all reagents used were from Sigma. IDE was immobilized on a COOH5 biosensor chip from ICxNomadics (Oklahoma City, USA). Covalent immobilization was obtained by amine coupling of the lysine-free amino groups and terminal amines of the protein, as described elsewhere.² Particularly, 400 μ L of IDE solution at 100 $\mu\text{g/mL}$ in 10 mM acetate buffer, pH 3.8, were used for the immobilization on a previously activated surface having reactive succinimide ester groups obtained by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) solution, freshly prepared (EDC = 0.4 M, NHS = 0.1 M). NaOH or HCl 1 mM were used for adjusting the pH of the buffer used. The latter was obtained by mixing N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) and sodium salt (0.01 M HEPES, 0.15 M NaCl, pH 7.4). The OneStep approach³ was applied to investigate the interactions between immobilized IDE and Ub whose initial concentration was 100 μ M. The software Qdat was used to fit the experimental curves according to the equations reported elsewhere.⁴ Briefly, the signal response (R) versus time (t) is given by:

$$dR/dt = k_a \cdot C \cdot R_{\max} - (k_a \cdot C + k_d) \cdot R$$

where, C is analyte concentration, Rmax is capacity of immobilized ligand, and k_a , k_d are the kinetic constants. For an injection beginning at time t_0 and ending at t_1 , we have:

$$c_1 = C \cdot k_a + k_d$$

For $t_0 < t < t_1$ (association phase):

$$R(t) = C \cdot k_a \cdot R_{\max} \cdot (1 - \exp(-c_1 \cdot (t - t_0))) / c_1$$

For $t > t_1$ (dissociation phase):

$$R(t) = C \cdot k_a \cdot R_{\max} \cdot (1 - \exp(-c_1 \cdot (t_1 - t_0))) / c_1 \cdot \exp(-k_d \cdot (t - t_1))$$

For multi-site kinetics, the total response is given as the sum of response from each individual site. So for site 1: $R_1(t, k_{a1}, k_{d1}, R_{\max1})$ and site 2: $R_2(t, k_{a2}, k_{d2}, R_{\max2})$, total response $R(t) = R_1 + R_2$. Fitting is performed using standard Levenberg-Marquardt nonlinear least squares optimization, minimizing the sum-squared error of the difference between the model and the experimental data

Molecular modeling. Docking simulations have been performed using HADDOCK interface.⁵ The starting coordinates of Ub were considered from the X-ray structure of the complex between the UBA1 enzyme and Ub (pdb code: 3CMM). The following residues of Ub were considered as active residues, since observed through NMR experiments to interact with IDE. Those include Q2, F4, L15, I44, F45, D52, V70, R72, R74. The binding surface of IDE reported in the X-ray structure (pdb code: 3OFI) was considered as active surface. Structures underwent rigid body energy minimization, semirigid simulated annealing in torsion angle space, with a final clusterization of the results.

Structural alignment of Molybdopterin biosynthesis protein (MoeB) with Insulin Degrading Enzyme (IDE).

The structural alignment of the two proteins MoeB and IDE was performed by using the flexible FATCAT tool present in the protein data bank : <http://www.rcsb.org/pdb/secondary.do?p=v2/secondary/analyze.jsp#Sequence>

FATCAT starts by identifying a list of AFPs (aligned fragment pairs)—a superposition of two continuous fragments—in the two proteins to be compared. The FATCAT structure alignment is formulated as an AFP chaining process, allowing flexibility in connecting them. The significance of the similarity detected by FATCAT is evaluated by a P-value that measures the chance of getting the same similarity in two random structures. This P-value is calculated based on the empirical fitting of the statistical distribution to the FATCAT similarity scores of the AFPs. The smaller the P-value, the more statistically significant the similarity. The smaller the P-value or the higher is the score, the more statistically significant the similarity between corresponding structures.

Scheme 1: Structural alignment of MoeB (pdb code 1JWA) and IDE (pdb code 2JG4) in text format, in which different measurements of the alignment are shown and alignment blocks are labeled incrementally from 1

```
Align 1JWA.B.pdb 217 with 2JG4.A.pdb 961
Twists 5 ini-len 176 ini-rmsd 6.61 opt-eu 174 opt-rmsd 4.35 chain-rmsd 20.81 Score 179.77 align-len 279 gaps 105 (37.63%)
P-value 7.34e-01 Afp-num 69749 Identity 3.94% Similarity 12.54%
Block 0 afp 5 score 64.24 rmsd 7.10 gap 30 (0.43%)
Block 1 afp 2 score 21.27 rmsd 3.44 gap 3 (0.16%)
Block 2 afp 5 score 66.08 rmsd 6.34 gap 7 (0.15%)
Block 3 afp 4 score 59.19 rmsd 4.87 gap 8 (0.20%)
Block 4 afp 1 score 20.31 rmsd 1.57 gap 0 (0.00%)
Block 5 afp 5 score 84.68 rmsd 5.33 gap 6 (0.13%)

MoeB :      2  AELSDQEMLRYNRQIILRGFD-----DGQEALKDSRVLIVGLGGLGCAASQYLASA
      1111      111111111      111111111111111111 111111111111
IDE   : 580  SPFAYVDPLHCNMAYLYLELLKDSLNEYAYAAELAGLSYDLQNTIYGYLSVKGYND-KQPILLKKIIEK

MoeB :      54  GVGNTLLDFDTVSLSNLQRQTL---HSDATVGPQKVESARDALTRINPHIAIT---PVN---ALDDAE
      11  22222222      333333      333333333333333333 333 33333333
IDE   : 649  MA--TFEIDEKRFEIIEKAYMRSLNNFRAEQPH----QHAMYRLRLMTEVAWTKDELKEALDDVTLPK

MoeB :     115  LAALIAEH-DLVLDCTD-----NVAVRNQLNAGCFAAKVPLVSGAAIRMEGQITVFTYEAG-----
      33333333 44444444      44444444444444444444 55555555555
IDE   : 712  LKAFIPQLLSRLHIEALLHGNITKQAALGIMQMVEDTLIEHAHTKPLPS-QLVRYREVQLPDRGWVFYQ

MoeB :     192  -----VMAPLIGVIGSLQAMEAIKMLAGYKGPASGKIVMYDAMTCQFREM
      66666666666666666666666666666666 66666 66666666
IDE   : 781  QRNEVHNHNCGIEIYYQTDQMSTSENMFLELFCQIIEPCFNTLRTEQLGFIVFSGPRRANGIQGLRFI
```

Note: positions are from PDB; the numbers between alignments are block index



Figure S1. 3D visualization of superposition between structure of IDE (red) and MoeB (yellow).

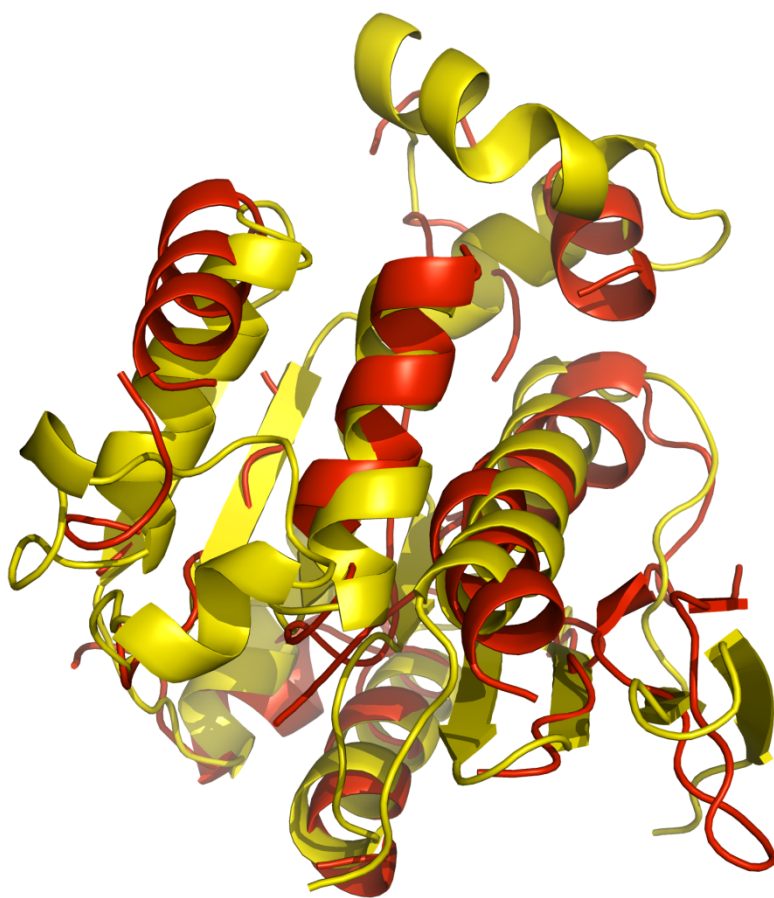


Figure S2. Visualization of superposition between structure of IDE Residues (580-850) (red) and MoeB (yellow).

Surface Plasmon Resonance

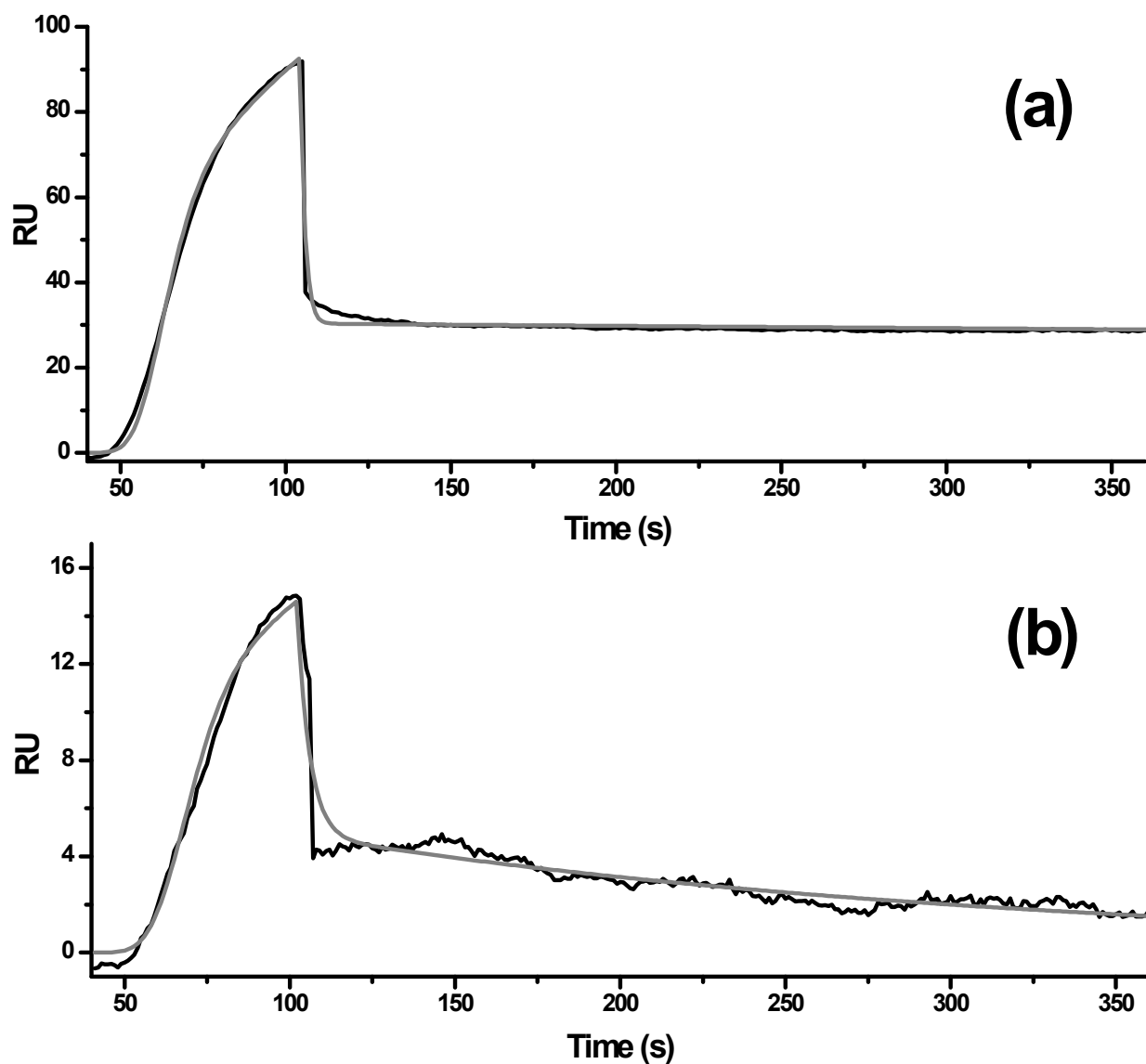


Figure S3: Experimental SPR response (black curve) and theoretical two binding sites fittings (grey curve) obtained for the interaction between immobilized IDE and ubiquitin (100 μM) without (a) and with (b) EDTA by the OneStep approach. The kinetic parameters obtained from the fittings are reported in Table S1. Note the lower affinity of ubiquitin toward EDTA inhibited IDE, discussion is in the text.

Table S1: Kinetic parameters obtained from the fitting of the SPR curves reported in Figure S3. The two binding sites model was necessary in order to obtain a good fitting (low Res sd value).

Without EDTA	k_a	k_d	R_{max}	K_D	Res sd
Site 1	70	$1.8 \cdot 10^{-4}$	130	3 μ M	1.510
Site 2	$5.36 \cdot 10^3$	0.69	143	130 μ M	1.510
With EDTA	k_a	k_d	R_{max}	K_D	Res sd
Site 1	80	$4.53 \cdot 10^{-3}$	20	60 μ M	0.552
Site 2	$2.0 \cdot 10^2$	0.258	150	1.3 mM	0.552

NMR spectroscopy

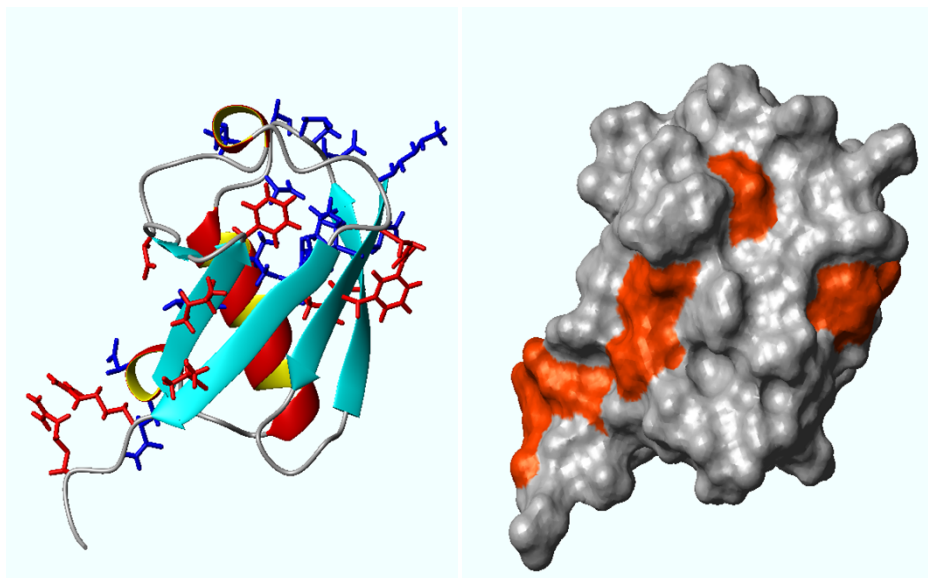


Figure S4: Mapping onto ubiquitin structure of intensity variations obtained after the addition of IDE defining the site of interaction. The residues whose peaks were reduced in intensities are reported in dark orange.

Western blot analysis of the performance of IDE as an E2 Ub-conjugating enzyme.

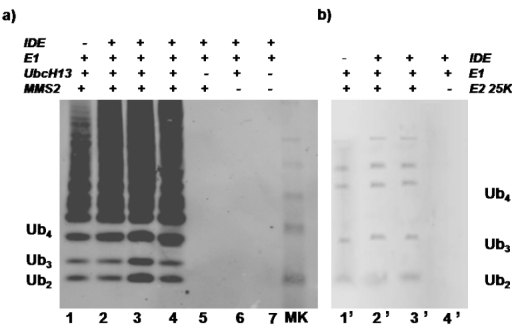


Figure S5. IDE is not an E2-like ubiquitin conjugating enzyme in self-ubiquitination reactions. a) line1: Ub (5 μM), UBE1 (100 nM), DTT (500 μM), MgATP (5 mM) UbchH13 (500nM) MMS2 (500nM) in ubiquitinylation buffer (MOPS 50 Mm pH 7,2);line2,3,4: 1+IDE (1; 5;10 μM); line5,6,7: 1 + IDE 1 μM instead of MMS2 or/and UbchH13. b) line1: Ub (10 μM), UBE1 (500 nM), DTT (500 μM), MgATP (5 mM) E2-25K (1 μM) in ubiquitinylation buffer (MOPS 50 Mm pH 7,2); line2,3: 1+IDE (1; 10 μM); line 4: 1 + IDE 1 μM instead of E2-25K.

References

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