

Two-stepping with E1

Andrew P. VanDemark and Christopher P. Hill

Covalent attachment of ubiquitin-like proteins to other proteins drives numerous important physiological processes. The recent structure of an ubiquitin-like E1 enzyme provides insight into the curious assembly line-like mechanism that initiates all ubiquitin-related protein processing pathways.

Ubiquitin is a small but well-studied protein. The ligation of ubiquitin to many different proteins targets these substrates for degradation by the 26S proteasome¹; the targeted degradation process then mediates a wide range of physiological responses². Protein ubiquitylation also has nonproteasome-associated roles, including endocytosis of cell surface receptors³, endocytic sorting⁴, DNA repair^{5,6}, inflammatory responses⁷ and budding of HIV⁸. To add to the complexity, a number of ubiquitin-like proteins, known as Ubls or Ublps, also exist^{9–11}. Attachment of these proteins to various substrates serves to regulate functions involved in, for example, autophagy, cellular morphology, signaling pathways, cell division, transcriptional activity and nuclear transport. Despite this remarkable functional diversity, ubiquitin and the other Ubls all seem to be mobilized and ligated by distinct yet parallel pathways consisting of related enzymes^{9–12}.

The ligation of Ubls to their many substrates is performed by pairs of ubiquitin-conjugating (E2) and ligating (E3) enzymes. Many different E2 and E3 enzymes exist in the cell because each pair can recognize and modify only a subset of the vast number of different substrates^{12,13}. In contrast, the entrance of Ubls into these ligation pathways is performed by ubiquitin-activating (E1) enzymes, and, in general, there is just one E1 enzyme for each different Ubl^{10,11,14,15}. For ubiquitin itself, the E1 is a single-chain protein of 110 kDa whose sequence displays a weakly conserved two-fold repeat. For many of the other Ubls, the E1 is a heterodimer where each subunit corresponds to one half of a single-chain E1.

E1 enzymes activate their respective Ubls by a two-step process^{16,17}. ATP is first hydrolyzed to bring about adenylation of the Ubl C terminus; Ubl is then covalently joined to a conserved cysteine side chain of E1 *via* a thioester bond. An assembly line-like process ensues, repeating the first step of adenylation on a second Ubl mole-

cule to produce a fully-loaded E1 bearing two Ubls, one in the form of an adenylate and the other as a thioester. At this point, other enzymes in the pathway become involved, and the Ubl covalently attached to E1 is transferred to form a thioester complex with an E2 enzyme, followed by eventual transfer to a target protein.

Study of the mechanism of E1 enzymes gained solid footing with the recently reported crystal structure of the E1 enzyme for Nedd8 (ref. 18), which appeared in *Nature*. Nedd8 (known as RUB1 in yeast) is a Ubl that is ultimately ligated to subunits of some ubiquitin ligases, thereby stimulating their activity^{19,20}.

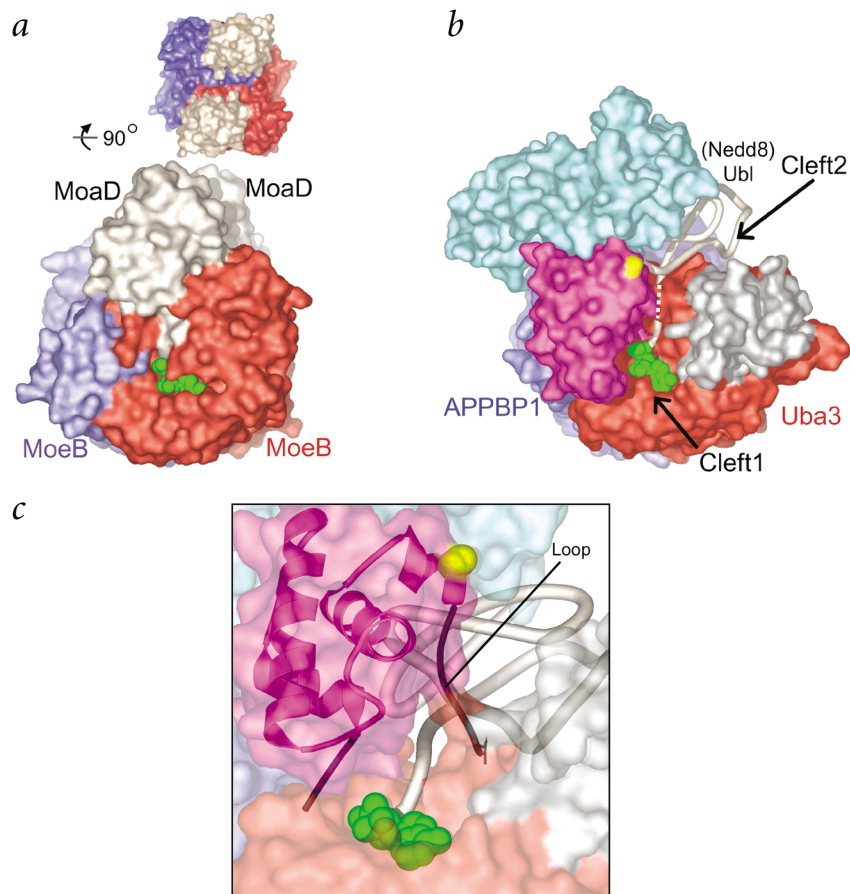


Fig. 1 MoeB-MoaD and E1 structures. **a**, MoeB-MoaD²³ shown as a surface representation. The MoaD C terminus is adenylated (green) in this product complex. The two-fold axis is vertical in the main panel, while the inset figure (above) shows a top view looking directly along the two-fold axis. **b**, Crystal structure of APPBP1-UBA3 with modeled Nedd8 adenylate. Orientation is the same as for MoeB-MoaD in (a). Adenylation domain segments of APPBP1 and UBA3 are colored to match the two MoeB subunits. Segments that form the catalytic cysteine domain are colored cyan (APPBP1) and purple (UBA3). Ubiquitin-like domain of UBA3 is gray, and the catalytic cysteine is yellow. The Nedd8 (white worm) and adenylate moiety (green) were positioned by superposition of the MoeB-MoaD complex on the E1 adenylation domains. The C-terminal residues of Nedd8 are shown as a dashed line where they are thought to pass through the crossover loop. **c**, Close up view of (b). The Nedd8 C terminus is shown passing through the crossover loop. Residues that connect the UBA3 adenylation and catalytic cysteine domains, including the crossover loop (loop), are shown in a darker color.

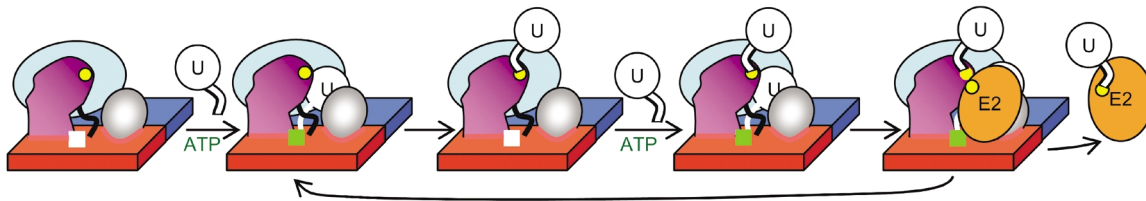


Fig. 2 Cartoon representation of the E1 reaction. The second step of transfer from adenylate to the catalytic cysteine avoids the potential topological trap. Color scheme is as for Fig. 1.

Clues from a distant relative

Although Ubl post-translational modification pathways are widespread in eukaryotes, they appear to be absent from prokaryotes. However, analogous processes are found in prokaryotes¹⁰, with one of the best characterized being the highly conserved synthetic pathway for the molybdenum cofactor (Moco)²¹. The Moco biosynthetic pathway involves adenylation of the C terminus of the Moad protein by an enzyme called MoeB. In a parallel with formation of the thioester-linked E1-Ubl complex, the C terminus of Moad is also transferred from an adenylate to a carbon-sulfur bond, but in this case with just a sulfur atom to yield a thiocarboxylate. An interesting difference is that the C terminus of Ubl is subsequently transferred from the thioester bond and the E1 cysteine side chain is released, whereas Moad thiocarboxylate transfers the sulfur to a new covalent bond within the molybdopterin component of Moco and releases the free Moad C terminus. Nevertheless, the observation that Moad adopts a ubiquitin-like fold²² lends further emphasis to the similarity between Moco biosynthesis and Ubl pathways.

Some time ago, Lake and co-workers²³ reported crystal structures of the two-fold symmetric MoeB–Moad complex (Fig. 1a). These structures suggested that catalysis involves a Mg²⁺ ion bound transiently to a conserved aspartate side chain, and that adenylation proceeds by direct attack of the Ubl C terminus on the ATP α phosphate. The MoeB–Moad structures also suggested a model for the adenylation domain of E1 enzymes, which share ~22% sequence identity with MoeB, and for the interaction between the E1 adenylation domains and their Ubl proteins²³.

E1 structure and mechanism

The Nedd8 E1 heterodimer structure now reported by Walden *et al.*¹⁸ is consistent with findings from the MoeB–Moad structures. It is composed of two subunits (APPBP1 and UBA3) that correspond to

the N- and C-terminal halves of a single-chain ubiquitin E1, respectively²⁴ (Fig. 1b). The catalytic cysteine and residues implicated in the adenylation reaction are all contained within the UBA3 subunit, although residues from its APPBP1 partner approach the active site regions quite closely. APPBP1 and UBA3 are tightly associated through an interface that buries ~7,000 Å² of accessible surface area, with the greatest contribution (~4,700 Å²) from the adenylation domain.

Regions of the E1 structure not present in MoeB include the catalytic cysteine domain, which is formed by a large insertion in both APPBP1 and UBA3. UBA3 also contains an extra domain at its C terminus that adopts a ubiquitin-like fold. Despite the additional domains and the absence of Nedd8 or ATP from the APPBP1–UBA3 E1 structure¹⁸, the E1 adenylation domain and MoeB display a high degree of structural similarity, as predicted²³. Walden *et al.*¹⁸ also describe biochemical and mutagenic experiments that support the prediction that the geometry of Ubl binding and mechanism of adenylation is conserved.

E1 displays a large active site groove that is divided into two distinct clefts, with ATP apparently binding in cleft 1 and Nedd8 in cleft 2. Comparison with the MoeB–Moad structure indicates that the C-terminal residues of Ubl will pass through a hole in the structure that is formed by loop 7 of UBA3 (the ‘crossover loop’), which separates clefts 1 and 2 and connects the adenylation and catalytic cysteine domains of UBA3. Moad extends its C-terminal residues through the equivalent crossover loop of MoeB, although this loop was disordered in the MoeB structure²³. A notable difference is that the MoeB crossover loop is a relatively short connection between different segments of the adenylation domain, whereas the E1 loop contains an additional ~75 residues of the catalytic cysteine domain. Indeed, the catalytic cysteine is located just a few residues after the end of this loop (Fig. 1c). This geometry is analogous to substrate

binding by the ubiquitin C-terminal hydrolases, which also requires the flexible Ubl C-terminal residues to pass through a crossover loop^{25,26}.

Although the relationship with the MoeB reaction appears to explain how E1 performs the adenylation step, a number of other questions are only partially resolved. First, how is the second step of transfer to the catalytic cysteine of E1 achieved? This residue, Cys216 in UBA3, is located at the end of a helix that connects to the crossover loop. Remarkably, Cys216 lies ~30 Å from the site of adenylation in the crystal structure, indicating that a significant conformational change will be needed to allow Ubl transfer from the adenylate. Assuming that Ubl does not alter the way in which it binds to E1 during this process and therefore is not given the opportunity to diffuse away prior to thioester formation, conformational changes in E1 would have to move the catalytic cysteine by at least 10 Å, even allowing for possible rearrangement of the inherently flexible Ubl C-terminal residues.

Following thioester formation, the Ubl must be released from the initial binding site to accommodate a second incoming Ubl for adenylation, raising the question of how the thioester-bound Ubl contacts the E1. One extreme possibility may be the complete absence of specific non-covalent interactions between E1 and the thioester-linked Ubl, as specificity could be adequately enforced by the initial adenylation reaction and subsequent covalent attachment.

Finally, Walden *et al.*¹⁸ have demonstrated that deletion of the C-terminal 95 residues of UBA3, which sit primarily in cleft 1 and adopt a ubiquitin-like fold, causes a reduction in E2 binding. This begs the question of exactly how E2 is bound and whether this interaction mimics one of the several different ways in which E2 and E2-like proteins appear to bind ubiquitin^{27–29}. In short, the recently determined E1 structure leaves us with both a significant advance and ample motivation for further study.

Why two steps?

The new data allow a structural representation of the E1 reaction (Fig. 2). It is interesting to ponder the functional importance of the second step of this process. It is not immediately obvious why the E1-catalyzed reaction should include a second step (that is, transfer to the catalytic cysteine), rather than simply transferring the Ubl adenylate directly to the E2 enzyme. After all, the ubiquitin C terminus is fully activated in a chemical sense by the initial adenylation step, and MoeB performs only the equivalence of the first step by adenylating Moad^{21,30}. We suggest that Ubl E1 enzymes selected thioester formation involving their catalytic cysteine as a mechanism to accommodate the large E2 adduct. Whereas the Moad C terminus is never covalently attached to a large adduct, an E1 that catalyzed thioester formation between a Ubl at the adenylation site and an E2 in cleft 1 would fall in a topological trap, with E2 and Ubl stuck on opposite sides of the crossover loop. This trap is avoided, however, by transfer of the Ubl C terminus from the

adenylation site to the catalytic cysteine located at one end of the crossover loop. Thus, the greater structural and mechanistic complexity of the eukaryotic E1 enzymes, compared to their simpler MoeB-like counterparts, may be dictated by their need to accommodate the larger E2 substrate involved in successive reaction steps.

Andrew P. VanDemark and Christopher P. Hill are in the Department of Biochemistry, University of Utah School of Medicine, 20 N 1900 E, Room 211, Salt Lake City, Utah 84103 USA. Correspondence should be addressed to C.P.H. e-mail: chris@biochem.utah.edu

- Hershko, A. & Ciechanover, A. *Annu. Rev. Biochem.* **61**, 761–807 (1992).
- Peters, J.M., Harris, J.R. & Finley, D. *Ubiquitin and the Biology of the Cell* (Plenum, New York; 1998).
- Hicke, L. *Trends Cell Biol.* **9**, 107–112 (1999).
- Katzmann, D.J., Odorizzi, G. & Emr, S.D. *Nat. Rev. Mol. Cell Biol.* **3**, 893–905 (2002).
- Spence, J., Sadis, S., Haas, A.L. & Finley, D. *Mol. Cell Biol.* **15**, 1265–1273 (1995).
- Hofmann, R.M. & Pickart, C.M. *Cell* **96**, 645–653 (1999).
- Deng, L. *et al. Cell* **103**, 351–361 (2000).
- Pornillos, O., Garrus, J.E. & Sundquist, W.I. *Trends Cell Biol.* **12**, 569–579 (2002).
- Hochstrasser, M. *Science* **289**, 563–564 (2000).
- Hochstrasser, M. *Nat. Cell Biol.* **2**, E153–E157 (2000).
- Jentsch, S. & Pyrowolakis, G. *Trends Cell Biol.* **10**, 335–342 (2000).
- Pickart, C.M. *Annu. Rev. Biochem.* **70**, 503–533 (2001).
- VanDemark, A.P. & Hill, C.P. *Curr. Opin. Struct. Biol.* **12**, 822–830 (2002).
- Zacksenhaus, E. & Sheinin, R. *EMBO J.* **9**, 2923–2929 (1990).
- McGrath, J.P., Jentsch, S. & Varshavsky, A. *EMBO J.* **10**, 227–236 (1991).
- Haas, A.L., Warms, J.V., Hershko, A. & Rose, I.A. *J. Biol. Chem.* **257**, 2543–2548 (1982).
- Haas, A.L. & Rose, I.A. *J. Biol. Chem.* **257**, 10329–10337 (1982).
- Walden, H., Podgorski, M.S. & Schulman, B.A. *Nature* **422**, 330–334 (2003).
- Hochstrasser, M. *Genes Dev.* **12**, 901–907 (1998).
- Read, M.A. *et al. Mol. Cell Biol.* **20**, 2326–2333 (2000).
- Leimkuhler, S., Wuebbens, M.M. & Rajagopalan, K.V. *J. Biol. Chem.* **276**, 34695–34701 (2001).
- Rudolph, M.J., Wuebbens, M.M., Rajagopalan, K.V. & Schindelin, H. *Nat. Struct. Biol.* **8**, 42–46 (2001).
- Lake, M.W., Wuebbens, M.M., Rajagopalan, K.V. & Schindelin, H. *Nature* **414**, 325–9 (2001).
- Osaka, F. *et al. A new NEDD8-ligating system for cullin-4A. Genes Dev.* **12**, 2263–2268 (1998).
- Johnston, S.C., Larsen, C.N., Cook, W., Wilkinson, K.D. & Hill, C.P. *EMBO J.* **16**, 3787–3796 (1997).
- Johnston, S.C., Riddle, S.M., Cohen, R.E. & Hill, C.P. *EMBO J.* **18**, 3877–3887 (1999).
- Miura, T., Klaus, W., Gsell, B., Miyamoto, C. & Senn, H. *J. Mol. Biol.* **290**, 213–228 (1999).
- VanDemark, A.P., Hofmann, R.M., Tsui, C., Pickart, C.M. & Wolberger, C. *Cell* **105**, 711–720 (2001).
- Pornillos, O. *et al. EMBO J.* **21**, 2397–2406 (2002).
- Leimkuhler, S. & Rajagopalan, K.V. *J. Biol. Chem.* **276**, 22024–22031 (2001).