

UBIQUITIN AT FOX CHASE

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by

IRWIN ROSE

University of California, Department of Physiology and Biophysics,
College of Medicine, Irvine, CA 92697-4560, USA.

My interest in protein breakdown as a research problem began in 1955 at about the time I joined the Biochemistry Department of Yale University. It was known that proteins break down intracellularly in the mature animal^{1, 2}. In 1955 I learned from Melvin Simpson about experiments he had published 2 years earlier³ showing that a variety of conditions that should lower the ATP level of liver slices (anaerobiosis, cyanide, 2, 4-dinitrophenol) decreased the rate of liberation of labeled methionine from protein of rat liver slices. Simpson and I had just joined the Biochemistry Department and we had down the hall labs from each other. Simpson's research goal at Yale was to look for an *in vitro* system that performed protein synthesis rather than protein breakdown. During my Yale years I concentrated on enzyme mechanism questions relating to the keto-aldoase isomerases using tritium to establish the occurrence of proton transfer⁴ but I kept an eye out for reports in the literature on progress in the protein breakdown field.

Nothing new was being reported by 1963 when we relocated to the Institute for Cancer Research of Fox Chase, Philadelphia. At that time I began looking for a cell free system that would show an ATP dependence using the Ehrlich ascites cells that were available from our animal colony. My usual procedure was to label cells in suspension with an essential amino acid, wash the cells, lyse them by homogenization, and look for acid soluble counts adding either ATP or 2-deoxyglucose plus hexokinase to deplete the endogenous ATP.

During 1972 on a half year sabbatical divided between Oxford (J. Knowles) and Jerusalem (Y. Stein), I was able to do further experiments with tissues supplied by Hans Krebs and by Jacob Mager, who had been Avram Hershko's Ph.D research director at the Hadassah Medical School. Both men were highly supportive. But I was not able to find a cell free system. At the same time I was talking to Jacob Bar Tana of the Biochemistry Department and we came up with a Pulse/Chase method that could be used to determine the functionality, binding constant and rate of dissociation of potential enzyme-substrate complexes⁵.

GETTING IT ALL TOGETHER

Hershko had been with Gordon Tompkins in San Francisco on a post doctoral fellowship essentially confirming Simpson's findings but with the tyrosine aminotransferase of cells in culture. When he returned to Israel to set up his own lab in Haifa he also looked for a cell free system. I did not meet Avram Hershko until 1975 at a Fogerty Conference on Regulation in Bethesda where we learned of each others interest in protein breakdown. Meanwhile Tompkins died tragically during a brain tumor operation. And Hershko was looking for a U.S. lab in which to expand his further work during sabbatical years and summers. It is not clear to me why he chose our lab in suburban Philadelphia for this purpose. We had no reputation in protein breakdown having never published in the field. Our limited expertise was expected to be in mechanistic enzymology.

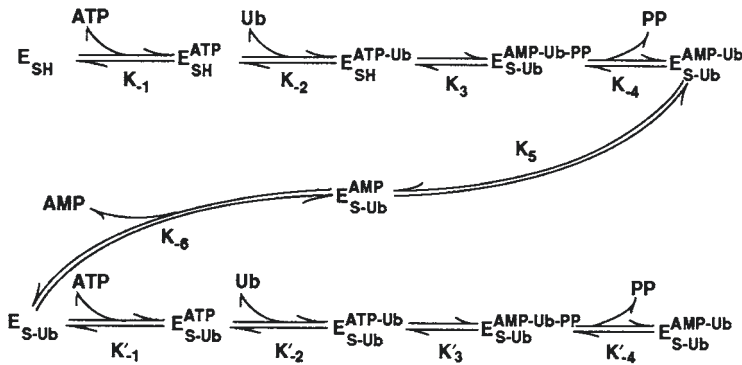
In 1977, when we joined forces at Fox Chase, Etlinger and Goldberg⁶ had done the first successful experiment using a cell free system with lysates of rabbit reticulocytes. Hershko and his student Aaron Ciechanover⁷ had already started fractionating the reticulocyte extract and recognized the heat stable factor (APF-1) before they came to Fox Chase that summer. Their subsequent work, much of which was reported in collaboration with members of the Rose lab, is to be found in the Hershko chapter. The key observation that 8 kDa APF-1 formed units of a chain linked to the protein targeted for breakdown was the most unexpected and unique aspect of the process⁸.

The identification of APF-1 with ubiquitin was made by three post docs at Fox Chase: Keith Wilkinson and Arthur Haas from the Rose/Hershko lab and Mike Urban from the next-lab over. Urban was working with chromatin and especially knew about their interesting components, the histones⁹. The pivotal question asked of Urban was: Do you know any examples of two proteins that are linked covalently. This recalled the small protein of unknown function, ubiquitin, a covalent ligand of histone H2A. The size and amino acid composition of APF-1 reported by Hershko and known for ubiquitin were in agreement.

The failure of many cell extracts to give ATP dependent protein breakdown is probably due to a lysosomal trypsin-like protease that destroys the ubiquitin of the preparation. This was discovered by Haas *et al.*¹⁰ who observed the loss of enzyme binding capacity of an ubiquitin affinity column when liver extracts were put through the column. Haas could show ATP/ubiquitin dependent protein breakdown with liver extracts that were preincubated to inactivate the trypsin-like activity. Ubiquitin was already known to be trypsin sensitive at the arg⁷⁴-gly⁷⁵ bond.

THE UBIQUITIN ACTIVATING ENZYME

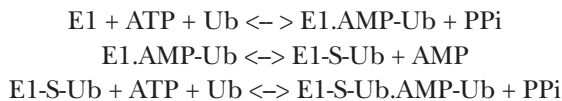
Enzymologists usually study the initial rates of reactions measuring product formation as a function of substrate concentration or other variable. Cell biologists are more likely to want to know the effect of a change on the steady



Scheme 1. The sequence and distribution of enzyme intermediates (Haas & Rose, 1982).

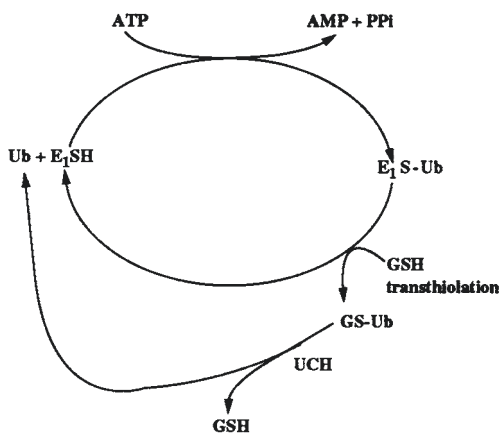
state behavior of a complex system. When the ubiquitin activating enzyme, E1, was discovered by Hershko it could not be studied by rate of product formation because the enzyme produced a covalently linked end-product. In 1982, Art Haas used isotope exchange at equilibrium to establish the reaction sequence and a number of equilibrium and rate constants of E1, the only enzyme of the cell that uses Ubiquitin, *per se*, as a substrate.

The Ub activation process is defined by the formation of two equivalents of PPi, one equivalent of bound AMP-Ub and two exchange reactions, ATP with PPi and ATP with AMP:



AMP-Ub, prepared from E1, ATP, and Ub and eluted by denaturing the enzyme is sensitive to both DTT and hydroxylamine indicating an acyl-P anhydride linkage. The AMP-Ub could be converted back to ATP upon addition of PPi and Mg^{2+} or to E-S-Ub which did not require PPi or Mg^{2+} ¹¹⁻¹³. Only the formation of enzyme-bound Ub was inhibited by iodoacetamide indicating transfer to cysteine of the enzyme, (Ub being cysteine free). The linkage to Ub had already been established to involve the C-terminal glycine of Ub¹⁴.

ATP was shown to precede Ub in combining with E1 and with E1-S-Ub (see Scheme 1) since Ub above 10 μ M caused inhibition of ATP/PPi exchange and at 400 μ M inhibition was complete. Addition was not random¹². Equilibrium constants of the expanded Scheme I could be estimated from



Scheme 2.

the influence of varying AMP and PPi on the concentrations of EAMP-Ub and ES-Ub which were determined with T-ATP by acid precipitation and labeled Ub by electrophoresis. The affinity for ATP, $\sim 40 \mu\text{M}$, tighter than the level of ATP in the cell assures that in the cell's E1 will be in the E1-ATP or E1-S-Ub-(ATP) form ready to pick up any free Ub ($K_m=0.58 \mu\text{M}$). The sensitivity to AMP as an inhibitor, $K_6=0.027 \mu\text{M}$, much tighter than ATP as a substrate, suggests that AMP may be acting as a feedback inhibitor at an allosteric site as well as with E-S-Ub.

UB-CARBOXY TERMINAL HYDROLASE: DISCOVERY AND MECHANISM

It was observed that the usual one turnover assay of E1 used ATP well beyond expectation when glutathione was included in the assay. The explanation: The Ub of E1-S-Ub is readily transferred nonenzymatically to mild nucleophiles such as glutathione, DTT, and hydroxylamine. By itself this would increase the consumption of ATP to the amount of Ub present in the assay. But much more was taken up. Were the Ub derivatives unstable? None of the expected Ub-S-DTT from an incubation of AMP-Ub and DTT could be isolated on a covalent-Hg⁺ column unless great care was taken in the conversion. Most revealingly the yield was increased if urea was added immediately after the reaction. This indicated that an enzyme carried over with the AMP-Ub from the E1 preparation was regenerating the Ub. These two observations: the transfer of Ub from E1-S-Ub to mild nucleophiles and an enzyme contaminating E1 that would restore free Ub were needed to explain why much more ATP was consumed than could have been expected in the E1 assay¹⁵. The combined

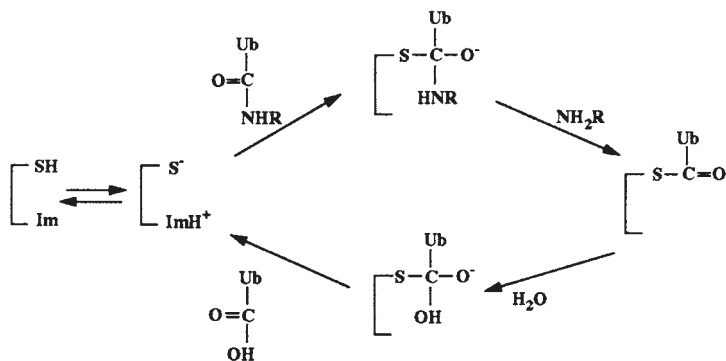
action of E1, glutathione, and the hydrolase results in a futile cycle converting ATP to AMP+PPi, Scheme 2. AMP-Ub is normally too tightly bound to E1 to lead to a futile cycle of its own in the presence of an active nucleophile.

Amides of Ub were not available to test as substrates of the new Ubiquitin-thioesterase until their synthesis was made possible by Cecile Pickart by action of E1+E2 on primary amines¹⁶. This was of great interest because the enzymes required for recovery of Ub from conjugation in the newly emerging Ub system were believed to be isopeptidases. An E1.E2-S-Ub complex normally transfers Ub to the ϵ -NH₂ of lysine of proteins. To determine the substrate specificity of this system Dr. Pickart found that a variety of small primary amines at much higher concentration are also very good Ub acceptors. The Ub-amides were good substrates for the previously purified Ub-thioesterase which henceforth has been known as Ub-Carboxy-terminal hydrolase, UCH. The pure enzyme, m.w. 29kDa, was readily prepared from mature human red cells (~0.1 Units/ml packed cells). Its turnover rate is quite high, almost diffusion limited and its V_{\max} is ~10 s⁻¹. Ub conjugated to glutathione or to a polyamine in cells with such activity should be negligible.

The rate of the E1.E2, UCH, amine system can be determined by measuring ATP consumption in a futile cycle similar to that of Scheme 2 where the rate can be used to test the activity or the specificity of the component present in rate determining amount. UCH enzymes are limited in the size of the substrate they will act upon. Any role in the deubiquitination of polyubiquitin chains is doubtful. This activity is given over to much larger enzymes, DUBs.

Considerations of the mechanism of UCH start with the observation that these enzymes are inactivated by iodoacetamide, protected by Ub, and therefore should have an active site thiol group and possibly a Ub-thioester intermediate. We found that inactivation of UCH was brought about by borohydride if ubiquitin was also present¹⁷. Both T from borohydride and label from Ub were tightly fixed to the inactive enzyme. Both isotopes were released upon mild acid denaturation. T traveled with the Ub. The released product was ~1000x more inhibitory than Ub in an assay using T-butanol-4-NH₂-Ub as a substrate. This effect was lost with borohydride addition to the inhibitor which was protected from reduction if enzyme were added first. We concluded that the acid-liberated inhibitor must be the C-terminal aldehyde form of Ub. This was shown with T-NaBH₄, and isolation of T-ethanolamine among the acid hydrolysis products. The basis for the protection of the Ubal by the enzyme must be the formation of a strong complex that shields the aldehyde function. A combination of chemical and physical forces would result from the addition of the active site -SH to the carbonyl, a thiohemiacetal on the one hand and multiple protein-protein interactions between the active site and the remainder of the Ubal. Additional stabilization may come from the resemblance of the thiohemiacetal to the tetrahedral intermediates and transition states in the amidase reaction as would be drawn for papain and cathepsin B, Scheme 3.

This interpretation of the mechanism of action of Ubal has been confirmed and extended by x-ray crystallography of Ubal complexed with the UCH



Scheme 3.

of yeast, Yuh 1 by Johnston *et al.*¹⁸ and with the 352 residue UCH domain that was cut out of the 1102 residue long HAUSP deubiquitinating enzyme (DUB) by Hu *et al.*¹⁹. In both cases Ubal caused significant distance and angle rearrangements in the catalytic triad regions compared with structures done without Ubal^{19, 20}, respectively as well as placement of H-bonding residues to accommodate the expected oxyanion hole of a thiohemiacetal Ub adduct.

The large DUBs, about 80 of which have been identified, serve to reverse the lengthening of the polyubiquitin chains that leads to the destruction of the targeted protein at the proteasome. HUASP is also known as a co-tumor suppressor protein because, by deubiquitinating p53, the tumor suppressor transcription factor of tissues, it should raise its steady state level. The large size of DUBs is consistent with high specificity and signal control. The roles of the UCHs are not yet clear. The size of the Ub-extension that can be removed by these smaller enzymes is smaller than a Ub so that Ub-Ub cleavage intermediates, if they arise in the deubiquitination process will not be further degraded by a UCH.

UNRESOLVED ISSUES

1. Current discussions of the E1 E2 E3 system often ignore the potential of E1-AMP-Ub to transport Ub units between the solvent and E1-SH to E2 without dissociation of the E1E2 complex. This possibility should be easily evaluated by pulse chase using heavy isotope labeled Ub in the pulse, unlabeled Ub in the chase and analyzing the product by mass spec sequencing.

2. This approach may fail if the E2.E3. protein interactions are weak. In

addition the experiment should give information about the processivity of the system, how many Ubs can be added in succession.

3. This approach should also answer the question of whether when Ubs are added to the growing chain they are added to the distally located end of the chain as is often stated but which seems unreasonable as the chain length increases.

4. An interesting problem stems from the observation by Cecile Pickart^{16,17} that hydroxylamine at $K_m \sim 1$ mM inactivates the C-terminal hydrolase of erythrocytes, and possibly all UCHs, in the required presence of Ub. Using the hydroxamate of Ub as a substrate, complete inactivation requires ~ 2000 turnovers of the enzyme. Labeled Ub is not found on the reisolated enzyme, nor is activity recovered. Unless hydroxylamine has some unexplored way of reacting with the S- of the Ub-thiolester- enzyme intermediate, the classical reaction products should be Ub-hydroxamate and active enzyme. In view of the important role of the deubiquitinating enzymes and a practical interest in their inactivation it will be useful to know how hydroxylamine works.

CREDITS

It is a pleasure to pay tribute to Dr. Avram Hershko whose analytic intuition was always productive and whose generous spirit made our interactions always harmonious. The post docs: Aaron Ciechanover, Art Haas, Cecile Pickart, and Keith Wilkinson were all major contributors who have continued their careers in the Ub field with notable success. Thanks are also due to Hannah Heller and Jesse Warms for their contributions in the lab.

Finally, we wish to pay tribute to Melvin V. Simpson who in 1953 demonstrated that protein breakdown in whole cells was energy dependent (3) and to Etlinger and Goldberg who in 1977 opened the way to *in vitro* studies with the demonstration of ATP dependent protein breakdown in rabbit reticulocyte lysates (6).

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