

But important questions arise from the short-term $\delta^{18}\text{O}$ -inferred temperature fluctuations at Kilimanjaro. In the tropics, the $\delta^{18}\text{O}$ values of rainfall exhibit a far stronger correlation with rainfall amount than with air temperature (13). The few detailed lake and stalagmite oxygen isotope records available in the East African–South Asian monsoon domain (9, 14, 15) have thus served as a proxy for variations in monsoon rainfall.

Recently, a postglacial oxygen record from diatom silica in some alpine lakes at Mount Kenya, supported by a well-constrained ^{14}C chronology (15), was interpreted by considering the factors governing the regional isotopic rainfall composition. The authors (15) concluded that centennial- to millennial-scale $\delta^{18}\text{O}$ fluctuations primarily reflect variations in moisture balance and cloud height driven by sea surface temperature anomalies over the tropical South Indian Ocean.

The Mount Kenya and Kilimanjaro isotope profiles show similarities (see the figure), but the marked $\delta^{18}\text{O}$ depletions at ~6500 to 5200 years before the present were

interpreted differently: Barker *et al.* argue that they reflect anomalously heavy snowfall (15), while Thompson *et al.* interpret them in terms of a substantial cooling (2).

The relative roles of temperature, water vapor trajectory, and precipitation amount on the tropical-montane isotope records thus remain controversial (15). Improving the global network for isotopic composition of precipitation (GNIP) (16) should resolve this question by helping to calibrate the tropical rainfall $\delta^{18}\text{O}$ composition in terms of climate parameters.

The unique ice core record of African climate presented by Thompson *et al.* (2) is also probably the last. The Kilimanjaro ice fields are shrinking fast in response to global warming, as are other tropical glaciers (3, 13). If the climatic trends of the 20th century continue, the ice on Kilimanjaro will disappear in the next 15 to 20 years (2). In the tropics, human societies suffer more from declining or irregular water resources than from changes in temperature. But global warming may have serious implications for local populations that depend on glacier meltwaters for

farming, irrigation, or hydroelectric power. There is also an urgent need to collect high-quality cores from tropical glaciers that will not preserve paleoclimatic archives for much longer.

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PERSPECTIVES: MOLECULAR BIOLOGY

New Proteases in a Ubiquitin Stew

Mark Hochstrasser

Some people like to say that they can tell the type of person you are by the company you keep. Many biologists regard this bromide as an effective guide to understanding intracellular proteins as well. Certain intracellular proteins are only active when built into larger protein complexes that coordinate multiple biochemical activities. The interactions between proteins in these complexes are frequently regulated in response to environmental stimuli or to changes in cell state, such as passage through the cell cycle. This regulation is most often mediated by reversible attachment of chemical modifiers, such as phosphate or acetyl groups. Polypeptides such as ubiquitin can also reversibly modify other proteins. Ubiquitin and ubiquitin-like proteins (Ubls) are enzymatically ligated to lysine side chains of protein substrates, forming amide (isopeptide) bonds. When attached as either monomers

or polymers, ubiquitin modifiers promote or inhibit binding of the modified protein to specific targets. Binding of polyubiquitinated proteins to the proteasome results in adenosine triphosphate (ATP)-dependent proteolysis of the protein substrate, with recovery of the intact ubiquitin tag.

Two studies—by Verma *et al.* (1) on page 611 of this issue and by Yao and Cohen in *Nature* (2)—demonstrate tight coupling between degradation and deubiquitination of protein substrates in the proteasome. Inactivation of the deubiquitinating enzyme (DUB) that releases ubiquitin chains from the proteasome completely prevents protein substrate degradation (1). Unexpectedly, the data suggest that the DUB is a proteasome subunit (Rpn11 or POH1) and may be a metalloprotease. The predicted protease motif, described in these papers and in an accompanying report by Cope *et al.* (3) on page 608 of this issue, has a widespread distribution in proteins from bacteria to mammals.

Cope *et al.* (3) analyzed an Rpn11-related protein, Csn5, which is part of another protein complex, the COP9-signalosome

(CSN) (4). The CSN is an eight-subunit heteromultimer similar in sequence and organization to a subparticle of the proteasome termed the “lid” (see the figure). Both Csn5 and the Rpn11 lid subunit must be incorporated into larger complexes before they are activated. Last year, the CSN was shown to promote the removal of a Ubl known as Rub1 or Nedd8 (here referred to as Rub) from a subunit of the SCF ubiquitin ligase, which is crucial for cell cycle progression (see the figure, A) (5). What had been puzzling about the earlier CSN study (5) was that every DUB and Ubl-cleaving enzyme identified in prior work had proved to be a cysteine protease. Yet no cysteine residue in any CSN subunit is both evolutionarily conserved and sensitive to mutation. In the new work, Cope *et al.* detect a His-X-His-X₁₀-Asp motif (the JAMM motif) in Csn5. The conserved amino acids in this sequence often coordinate metal ions in the active sites of hydrolytic enzymes. Cope *et al.* hypothesized that a Rub isopeptidase active site in the CSN is formed by this motif (together with a potentially important Glu residue further upstream). Subsequent biochemical and genetic experiments indicated that this motif is likely to be a metal-binding site necessary for cleavage of Rub from SCF.

Much more is known about the biochemical activities of the proteasome than of the CSN. The proteasome is a proteolytic machine that recognizes polyubiquitinated protein substrates, unfolds them, threads them

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through a narrow orifice into an interior proteolytic chamber, and releases their ubiquitin chains either before or after the initiation of proteolysis (see the figure, B). The new studies suggest that the removal of ubiquitin is essential, possibly because the proteasome is unable to unfold and translocate proteins linked to a chain of thermodynamically stable polypeptides.

The identity of the proteasomal DUB and how deubiquitination is integrated with other proteasomal activities has proved difficult to elucidate. In previous work, Verma and colleagues reconstituted the polyubiquitination and proteasomal degradation of a yeast cell cycle regulator, Sic1, using purified proteins (6). To their surprise, when they incubated ubiquitinated Sic1 with a proteolytically inactivated yeast proteasome, they obtained substrate devoid of polyubiquitin (1). Intriguingly, this DUB activity was ATP dependent and was not slowed by ubiquitin aldehyde (Ubal), an inhibitor of classical DUB enzymes. These features are reminiscent of a proteasome-associated DUB activity described many years ago but not further explored (7).

Meanwhile, Yao and Cohen (2) probed the mammalian 26S proteasome with a chimeric protein composed of a ubiquitin monomer fused to the amino terminus of an irreversibly denatured reporter protein. When ubiquitin cleavage from the substrate was prevented by mutating the junction between ubiquitin and the reporter protein, degradation of the fusion protein slowed by a factor of ~10 without a change in proteasome affinity. Thus, ubiquitin release from the substrate could be rate-limiting for degradation, presumably because of the difficulty in unfolding the ubiquitin domain, a necessary step before the polypeptide chain can slip through the narrow entryway into the proteasome core. If a tetraubiquitin chain was added to a lysine side chain in the amino-terminal ubiquitin of different fusion proteins, the resulting pentameric polyubiquitin unit was released en bloc upon incubation with 26S proteasomes and ATP. In contrast, if only the regulatory (19S) proteasome subcomplex

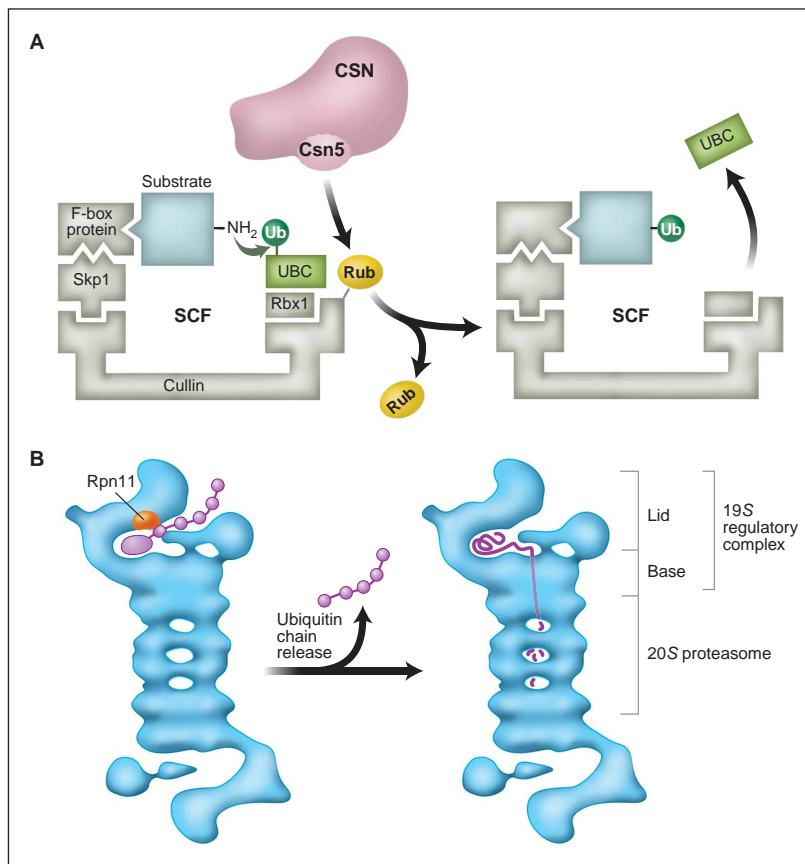
was used, Ubal-insensitive deubiquitination was no longer ATP-dependent. The simplest interpretation is that release of the polyubiquitin chain from the substrate is somehow coupled, in the 26S proteasome, to its ATP-dependent degradation.

The crucial question, then, concerns the identity of this mysterious proteasomal DUB. In light of the Cope *et al.* analysis of Csn5 (3), the related Rpn11 subunit of the protea-

Mutations in this putative metal-binding motif were lethal in yeast and led to proteolytic defects in vivo. Preincubation with a metal chelator blocked deubiquitination and degradation by purified proteasomes as well (1). A Zn^{2+} -specific chelator destroyed the DUB activity of the 19S regulatory complex, and addition of extra zinc ions restored it, suggesting that the metal bound by the His-X-His- X_{10} -Asp motif is zinc (2). Verma *et*

al. took the analysis a step further in vitro by using yeast proteasomes isolated from an *rpn11* heterozygous mutant; fortuitously, these proteasomes only contained the Rpn11 subunit bearing mutations in the His-X-His sequence. Degradation of their polyubiquitinated cell cycle substrate was completely blocked, as was polyubiquitin chain release. These data strongly suggest that substrate deubiquitination mediated by Rpn11 is necessary for substrate degradation, a conclusion congruent with the Yao and Cohen work. Tight coupling between substrate deubiquitination and degradation would enable the ubiquitin chain to target proteins to the proteasome but would ensure timely release of the chain so that full substrate unfolding and translocation into the proteasome core could proceed.

Strictly speaking, neither group has shown that Rpn11 is the actual DUB in these reactions: Zinc and a putative metal binding site are required for deubiquitination, but the motif could in principle be a binding site for another copurifying enzyme or a regulatory region for a catalytic site elsewhere in the proteasome. Although this seems unlikely, structural analysis with active-site inhibitors or chemical modification studies will be needed to show unequivocally where the catalytic centers lie. Another lingering question is how other proteasome-associated DUBs might contribute to ubiquitin release from the proteasome. Ubp6, for example, is a DUB that binds to the proteasome, and binding stimulates Ubp6 activity (8), suggesting at least an ancillary role in proteasome action.



Metalloproteases in large protein complexes. (A) The COP9-signalosome (CSN) catalyzes the removal of the Rbx1 ubiquitin-like protein from the SCF ubiquitin ligase and thereby modulates SCF ligase activity, probably by stimulating dissociation of the ubiquitin-conjugating enzyme (UBC). The yeast Sic1 protein is an example of an SCF substrate. (B) A polyubiquitin tag directs proteins to the proteasome, but it must be removed to allow degradation of the protein substrate. The lid component of the 19S regulatory complex of the proteasome is similar in composition and organization to the CSN.

some became an immediate candidate (1). Yao and Cohen (2) also pinpointed Rpn11 but by an independent route. They realized that Rpn11 was much better conserved than the other non-ATPase subunits of the proteasome 19S regulatory particle, suggesting that it might be an enzyme. Besides Csn5, Rpn11 is also related in sequence to several proteins not expected to have protease activity. Yao and Cohen looked for residues that were conserved between Csn5 and Rpn11 but not shared by the other proteins. The residues they identified included the His-X-His- X_{10} -Asp signature noted by Cope and colleagues.

There are many other proteins containing the predicted metal-binding motif, although none have been reported to be proteases. One is the human protein *c6.1A*. Chromosomal translocations involving the *c6.1A* gene have been linked to leukemia (9). Another example is AMSH (associated molecule with the SH3 domain of STAM) (10). STAM proteins are regulators of cell signaling and membrane protein trafficking. STAMs and related endocytosis proteins contain ubiquitin-interaction motifs that are necessary for the monoubiquitination of these proteins and for their normal function. If AMSH were a DUB, its ability to bind to the STAM SH3 domain might enable it to deubiquitinate

STAM or an associated ubiquitinated protein. Provocatively, the same SH3 domain in STAM also binds to a known DUB (UBPY), and both UBPY and AMSH use the same noncanonical SH3-binding motif for this interaction (10). Together, these data suggest a model wherein both classical and nonclassical DUBs modulate ubiquitin-dependent membrane sorting decisions, possibly at distinct steps in these pathways.

The picture that emerges from these latest studies is of an elaborate interplay between mechanistically diverse proteases that work at multiple stages of the ubiquitin pathway. These intricacies further emphasize the astonishing regulatory capacity of the ubiquitin

system and predict that ubiquitin, Ubls, and the enzymes that manipulate them will eventually be found to influence virtually every aspect of eukaryotic cell regulation.

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PERSPECTIVES: ENZYMOLOGY

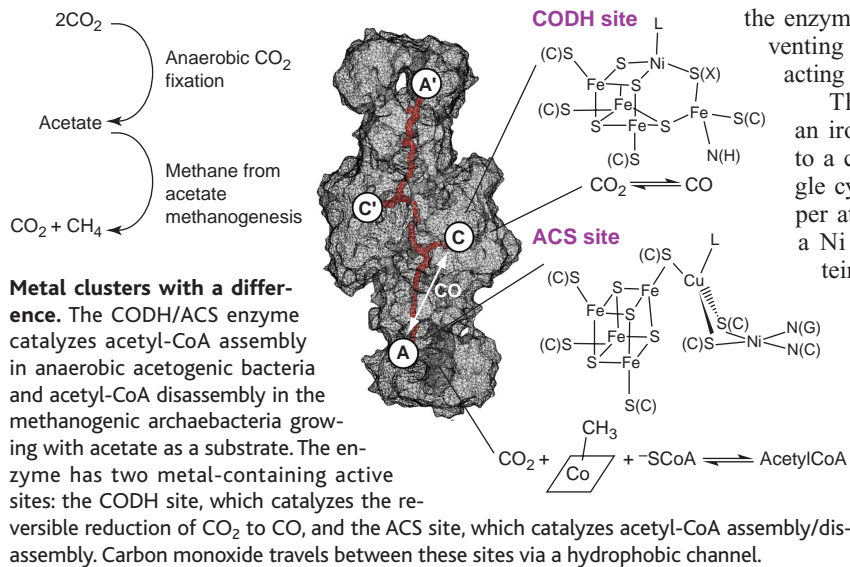
A Trio of Transition Metals in Anaerobic CO₂ Fixation

John W. Peters

Certain acetate-producing anaerobic microorganisms can synthesize biomass via the formation of acetic acid from carbon dioxide. A methyl group and carbon monoxide are derived from carbon dioxide and subsequently condensed with coenzyme A to generate biomass (1–3). On page 567 of this issue, Doukov *et al.* (4) report the crystal structure of the key enzyme in this process, carbon monoxide dehydrogenase/acetyl-coenzyme A synthase (CODH/ACS).

The structure reveals, much to the surprise of many investigators, a trio of transition metals at the active site: iron, nickel, and most unexpectedly, copper. Although clusters with two different transition metals are common, no metalloprotein active site with three different transition metal ions has been reported previously. The discovery also points to a new role for copper in biological systems.

In the acetate-production pathway, one CO₂ molecule is reduced to a methyl group in a set of enzymatic reactions using tetrahydrofolate. A second CO₂ molecule



Metal clusters with a difference. The CODH/ACS enzyme catalyzes acetyl-CoA assembly in anaerobic acetogenic bacteria and acetyl-CoA disassembly in the methanogenic archaeobacteria growing with acetate as a substrate. The enzyme has two metal-containing active sites: the CODH site, which catalyzes the reversible reduction of CO₂ to CO, and the ACS site, which catalyzes acetyl-CoA assembly/disassembly. Carbon monoxide travels between these sites via a hydrophobic channel.

is reduced to carbon monoxide at the CODH active site in the CODH/ACS complex. This active site, termed the C-cluster, contains a distorted cubane constructed from one nickel atom, three iron atoms, and four sulfur atoms bridged to an additional iron atom (5, 6).

Once formed, the methyl group and the carbon monoxide react with coenzyme A to form the product acetyl-coenzyme A (CoA). This complex reaction of coordinated carbon-carbon bond and carbon-sulfur bond formation occurs at the A-cluster. Methanogenic archaeobacteria use analogous reactions involving a similar set of cofactors and coenzymes in the reverse direction to disassemble acetyl-CoA, using acetate

as the growth substrate (see the figure) (7).

An amazing feature of the CODH/ACS enzyme is the hydrophobic channel that spans nearly the entire complex. This channel is ~13.8 nm long and is reminiscent of the channel in another enzyme, carbamoyl phosphate synthase (8). Doukov *et al.* suggest that the channel could allow the accumulation of carbon monoxide in the enzyme for catalysis while preventing the toxic gas from interacting with cellular components.

The A-cluster consists of an iron-sulfur cubane bridged to a copper ion through a single cysteine thiolate. The copper atom is in turn bridged to a Ni atom through two cysteine sulfur atoms. Enzymes with active sites containing complex bridged metal assemblies include nitrogenase, hydrogenase, and sulfite reductase (9, 10).

The coordination environments of both the copper and the nickel atoms in the A-cluster are interesting.

The copper atom is coordinated by three cysteine sulfur atoms, and the authors have tentatively assigned the fourth ligand to be an acetyl group or perhaps a carbonyl bound in multiple conformations representing an intermediate in catalysis. The nickel atom is in a square planar environment with thiolate and amide nitrogen coordination. The planar environment and amide coordination is reminiscent of the Ni porphyrinoid in methyl-coenzyme M reductase (11).

In their careful structural analysis, Doukov *et al.* used the anomalous scattering properties of the native metal ions in x-ray diffraction data collected near their absorption edges to assign the position

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