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Induced structure of a helical switch as a mechanism to regulate enzymatic activity

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Herpesviruses encode a protease that is activated by homodimerization at high enzyme concentrations during lytic replication. The homodimer contains two active sites, which are distal from the dimer interface. Assignment of backbone NMR resonances and engineering of a redox switch show that two helices position a loop containing catalytic residues within each active site.

Proteolysis has devastating consequences when unregulated but can serve as a powerful binary switch when controlled. Human herpesvirus 8 encodes a protease whose activity is required for viral lytic replication and virion formation[1–4]. A lack of structural information regarding the inactive monomer has prevented elucidation of the regulatory mechanism for this important class of potential therapeutic targets. The crystal structure of dimeric Kaposi's sarcoma–associated herpesvirus (KSHV) protease reveals that each monomer contains a spatially separate active site that is distal from the dimer interface, and it has been hypothesized that an undescribed conformational change regulates activity[5,6]. We set out to elucidate the molecular mechanism underlying enzyme regulation of dimerization.

A single point mutation at the interface of KSHV protease (M197D) generated an inactive monomeric variant of the enzyme. Both circular dichroism and NMR analysis have shown that the variant monomer is structurally indistinguishable from the wild-type monomer produced by heat-induced dissociation of the native enzyme[5]. This result, coupled with our finding that active site inhibition of the enzyme resulted in an extreme shift in the equilibrium toward the dimer[7], gave us the opportunity to separately analyze the structures of monomeric and dimeric enzymes to identify the native states that normally exist in dynamic equilibrium[7]. Because of the relationship between activity and dimerization, monitoring dimerization-dependent structural rearrangements provides insights into the mechanism of enzyme activation.

NMR measurements defined the structural transition upon dimerization, which represents a previously unobserved mechanism of protease activation. Triple-resonance experiments and amino acid–specific labeling resulted in assignment of all but one of the residues in helices 5 and 6, and of 94% of the total α-carbons. Amino acid–specific secondary structural information for monomeric KSHV protease was provided by analysis of the Cα chemical shifts[8–10], which, except in helices 5 and 6, showed a strong correlation with the dimeric crystal structure (**Fig. 1a**).

Hydrogen/deuterium exchange experiments confirmed the chemical shift analysis by showing that helices 5 and 6 unfold upon dissociation, resulting in 31% loss of α-helical structure (**Fig. 1b**). The inherent exchange rates calculated on the basis of primary sequence[11] cannot account for the differences in protection among the six helices. Therefore, the reduced protection of the two carboxyl helices is due to different helical stabilities.

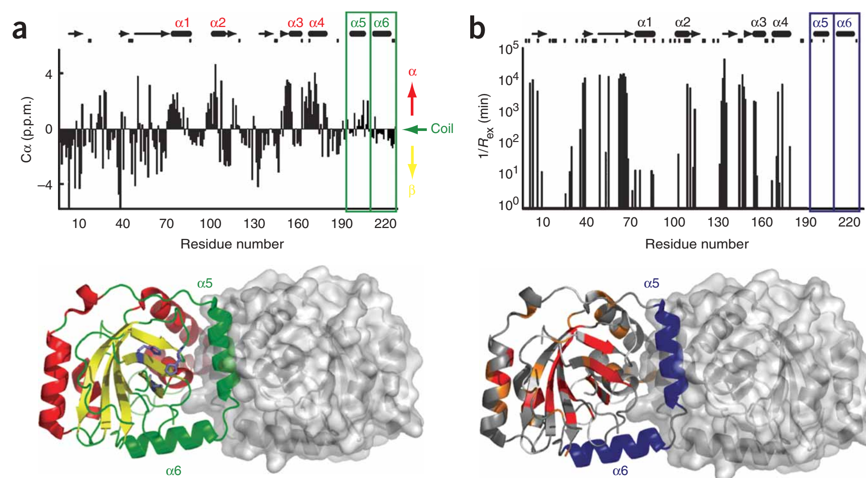

Figure 1 Structural rearrangement upon protease activation. (**a**) Top, NMR chemical shift index showing that helices 5 and 6 of the dimer are not present in the monomeric enzyme. Secondary structural elements (arrows, α-helices; bars, β-sheets) and unassigned residues (dots) are indicated above the graph. Bottom, NMR data pertaining to the monomeric form in solution mapped onto the dimeric crystal structure: yellow, sheet; red, helix; green, disordered loops. (**b**) Top, hydrogen/deuterium exchange rates corroborating instability of helices 5 and 6. Bottom, H/D exchange rates mapped onto the crystal structure: red, slow; orange, medium; gray, fast; blue, entire secondary structural elements lost.

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Viral enzyme activity seems to be regulated by a local 'helical switch' rather than by global unfolding. To support this mechanism, we engineered a carefully placed disulfide bond (G145C-V219C) that stabilized the interaction of helix 6 with the enzyme active site. Because wild-type enzyme has no disulfide bonds, incorporation of a cysteine into both helix 6 and the oxyanion loop allowed formation of the only productive intramolecular disulfide bond in the molecule. To prevent intermolecular disulfide bond formation, the surface cysteines of the endogenous KSHV protease were mutated to serines, without any appreciable effect on catalysis (data not shown). The enzyme's redox switch controls conformational rearrangement with concomitant regulation of activity. **Figure 2a** shows the cycle of activity of the engineered disulfide variant in oxidizing conditions (active), reducing conditions (inactive) and oxidizing conditions (active) again. Insets show the acquisition of dimeric quaternary structure with the attainment of enzymatic activity. These results indicate that activity is dependent on disulfide formation and on stabilization of the interaction between helix 6 and the active site loop. To further illustrate the importance of this interaction, the intramolecular disulfide bond was engineered into the inactive monomeric protease variant, resulting in the first rationally designed active monomer (**Supplementary Fig. 1** online).

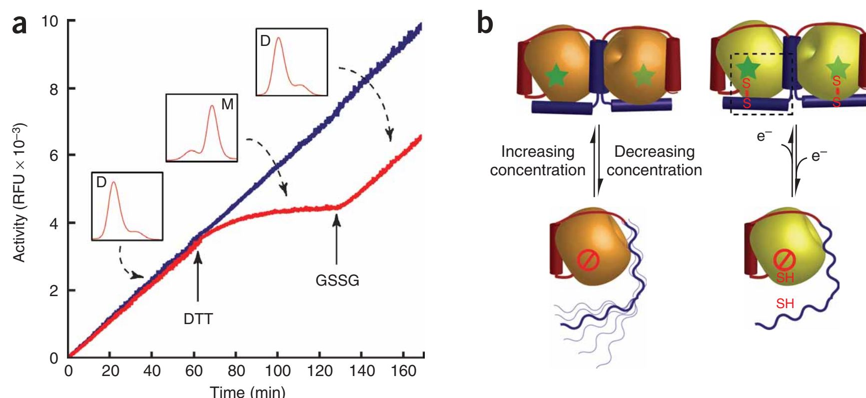

Figure 2 Proteolytic activity is controlled by a redox switch. (**a**) Activity of KSHV protease with surface cysteines removed (blue) and disulfide bond engineered (red), monitored by an increase in fluorescence upon cleavage of a fluorogenic substrate; RFU, relative fluorescence units. After 60 min, DTT (1 mM) was added to the sample and activity was monitored for an additional hour before addition of oxidized glutathione (GSSG; 10 mM). Insets, quaternary structures of oxidized (untreated), reduced and reoxidized protease, analyzed by analytical size exclusion chromatography at 280 nm; D, dimer; M, monomer. (**b**) Schematic of wild-type and redox-controlled protease activation. Dimerization results in assembly of a functional active site (star), whereas dissociation disrupts the catalytic site (null sign).

Although oligomerization has long been known to affect protein function, here we used NMR and protein engineering to determine the molecular mechanism of the unique oligomerization-dependent mechanism of regulation for herpesvirus proteases (**Fig. 2b**). Folding of helices 5 and 6 upon dimerization positions a loop that contains key components of the catalytic machinery required for transition-state stabilization during substrate hydrolysis. Arg142 and Arg143 provide hydrogen bonds shown previously to be essential for catalysis[12]. The oxidized, engineered disulfide at positions 145 and 219 stabilizes these components (**Supplementary Fig. 2** online).

Does the concentration-dependent acquisition of protein function described above have a biological role? On the basis of available data, we estimate that the concentration of protease in an infected cell increases from 5 nM in the cytosol to 100 μM in the immature capsid (**Supplementary Methods** online). This increase in protease concentration is greater than four orders of magnitude and should correspond to an increase from 1% to 93% active enzyme upon capsid formation, given the measured enzyme dissociation constant of 1.8 μM (ref. 13). We note that although the protease is expressed as a fusion protein, significant enhancement of its dimerization in the cytosol would result in premature activation of the protease, yielding no mature capsids. It seems that herpesvirus proteases have evolved a weak dimerization constant that reduces the amount of active protease in the cytosol and averts disruption of mature infectious particle formation. The negative entropy of helix folding represents a mechanism underlying the fact that, despite burying more than 2,000 Å^2 of hydrophobic surface area upon dimerization, herpesvirus proteases have only micromolar dissociation constants.

Multicomponent cellular processes must integrate many disparate enzymatic activities to ensure survival of the organism. Oligomeric complexes of proteins are ubiquitous, and their assembly offers a mechanism for controlling protein function. The large conformational rearrangements that occur in herpesvirus proteases upon dimerization regulate enzymatic function. This allosteric control allows the integration of proteolytic activity into the lytic cycle in a controlled manner and illustrates the large differences that exist between the isolated and oligomeric forms of a protein. We anticipate that this transitional helical switch is not unique to the regulation of enzymatic activity but may be applicable to other diverse protein functions as well, and may also be used in any chemical transition to provide control of biological processes through acquisition of quaternary structure.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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