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A protein crystal has been grown, which uniquely, is fully cross-linked by cysteine-mediated disulfide bonds along the c-axis.
A disulfide polymerized protein crystal

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The vEDD coiled coil domain from human BAP29 was crystallized in dimeric and tetrameric forms. For the dimer, a disulfide bond was unexpectedly found to bridge a crystal contact, resulting in complete cross-linking along the c-axis. This indicates that it is in principle possible to design spontaneously polymerizing protein crystals.

BAP29 is a 28 kDa protein belonging to the BAP31 family, which resides in the endoplasmic reticulum and is involved in regulating intracellular sorting of several membrane proteins. It consists of an N-terminal membrane-bound domain with three predicted transmembrane helices and a C-terminal cytoplasmic region containing a so-called vEDD domain (~7.5 kDa). X-ray structure determination has recently revealed that the vEDD domain of the closely related BAP31 folds as a parallel coiled coil dimer. CD spectroscopy data suggested that BAP29 vEDD likewise forms a coiled coil, but folding could only be detected at acidic and not at neutral pH under otherwise identical conditions. In order to confirm that human BAP29 vEDD forms a coiled coil and to gain insights into why low pH may favor folding of the domain, crystallization was carried out. However, this study does not only provide insights into the structure of BAP29 vEDD, as the crystals also exhibit unexpected properties of potential bio/nanotechnological relevance. Historically, protein crystals were first prepared as a means of purification, but are now mainly used for structure determination. However, cross-linked protein crystals have also been used as catalysts with higher stability and sometimes higher enantioselectivity than soluble enzymes, and can also be used as storage containers for small molecules, e.g. drugs, and as column material for chromatographic separation. Furthermore, with the advent of nanotechnology, applications are now also emerging as templates for synthesis of inorganic nanomaterials, and as functionalized reaction vessels created through specific coupling of small molecules within the solvent channels. Protein crystal engineering rests on some of the same principles as the much more established discipline of small molecule crystal engineering e.g. at the heart of both disciplines lies the concept of viewing the crystal as a supramolecular entity with emergent properties. However, protein crystals also pose their own specific advantages and challenges. One important challenge is that most of their applications require them to be cross-linked to overcome stability issues. New tools to facilitate this step could therefore greatly aid in moving the field forward.

In a broad screen for crystallization conditions, two crystal forms were obtained for BAP29 vEDD, both at pH <5 (see supplementary methods and supplementary table 1). These forms represent two different structural configurations: The expected parallel coiled coil dimer (2.5 Å max resolution, PDB: 4W7Y) and an unexpected antiparallel coiled coil tetramer (2.2 Å max resolution, PDB: 4W7Z) (Fig. 1a-b). Notably, in the dimeric form, E169 and E170 from chains A and B are in close proximity to each other near the N-terminus, suggesting that protonation of these residues may be required for folding (Fig. 1c). Furthermore, the tetramer interface is stabilized by several hydrogen bonds involving E176, E183, E190, E207, E218 and E225 of all four chains (Fig. 1d). The structures thus indicate that the pH sensitivity of folding is caused by a requirement for protonation of glutamates, which are close together in the folded state. The vEDD domain is exposed to the cytoplasm, which does not attain sufficiently low pH to support protonation of exposed glutamate side chains. The question therefore arises if these structures are...
physiologically relevant. The dimer resembles the structure of BAP31 vDED, which is stable at neutral pH, and it may therefore be speculated that it can also exist in vivo under certain conditions. Possibilities include e.g. that the charges of E169 and E170 can be compensated for by regulatory cations or proteins, or that under certain circumstance, the C-terminal region can fold by itself leaving the acidic N-terminus unfolded. On the other hand, the presence of a large number of glutamic acid-mediated hydrogen bonds over the entire interaction face of the tetramer, makes it quite unlikely that this form can exist in the cytoplasm, even if interacting with regulatory ions or proteins.

BAP29 vDED contains a single cysteine close to the N-terminus, C171 (Fig. 1a-b). In the dimeric crystal form, a disulfide bond is formed between C171 of chains A and B across a crystal contact, resulting in all dimers in the crystal being cross-linked along the c-axis (Fig. 2a). The electron density for the disulfide bond is strong and very well defined, and there are no indications of alternative side chain conformations (Fig. 2b).

There is furthermore no negative difference density, suggesting that radiation damage is negligible. The \( \chi_2 \) torsion angle around the -S-S- bond is -96\(^\circ\) and thus close to the often cited ideal value of \( \pm 90^\circ \) and within the normal range of disulfide torsion angles observed in proteins, although slightly different from the reported peak values of -87\(^\circ\) and 97\(^\circ\). The strength of the interaction is therefore probably comparable to that of naturally occurring disulfide bonds. The average \( B \) factor for the cysteine side chains is just 35.9, which is much lower than the average of 73.0 for all atoms of the protein. Furthermore, \( B \) factors are also lower in the area around the disulfide bonds than elsewhere in the protein, suggesting that they may add considerable stability to the crystal (Fig. 2c). The individual dimers are intercalated such that only single dimers or doublets of two disulfide bonded dimers can be added at the same time to a growing crystal. It is therefore clear that at least some and possibly all disulfide bonds form upon or after a new dimer is added to the crystal rather than being pre-formed in solution. Indeed, a reducing agent was present in the protein sample, which to some extend would hamper disulfide bond formation in solution. Interestingly, a dimeric coiled coil with a similar placement of a cysteine near the N-terminus has been shown to self-assemble into disulfide cross-linked fractal structures. No disulfide bonds are present in the high-resolution structure of the tetramer, but C171 of chains A and B are both close to a symmetry-related C171 (supplementary fig. 1). Indeed, a disulfide polymerized crystal could be obtained by omitting the reducing agent from the crystallant (max resolution of 3.2 Å, PDB: 4W80). However, the disulfide bonds of the tetramer are probably less useful for further development of disulfide cross-linked crystals than those of the dimer, as the geometry could not be as well characterized due to the rather low resolution of the data, and as they do not appear to decrease local dynamics in the crystal contacts where they occur (supplementary fig. 1). Several programs are available for designing disulfide bonds through site-directed cysteine mutagenesis. Furthermore, various approaches to protein crystal design have been developed. Indeed, a fully computationally designed coiled coil crystal has recently been reported. Taken together with this study, it therefore seems plausible that it may become possible to design protein crystals, which spontaneously form disulfide cross-links. In relation to using coiled coils for this purpose, it is noteworthy that the individual chains of dimeric coiled coils can also be cross-linked to each other through cysteine insertion in the dimer interface. Cross-linking is a prerequisite for most applications of protein crystals. It is currently achieved through post-crystallization soaking, but often requires considerable optimization and may be heterogenous/incomplete, decrease packing order or even destroy the crystal. Spontaneous polymerization through presynthesized disulfide bonds could therefore be an attractive alternative.

Based on the Matthews coefficient, the solvent content of the dimeric crystal form can be estimated to be 70\%, which is a very high value for a dimer with a subunit size of just 7.5 kDa. Furthermore, the solvent is localized almost entirely to a single 75x90 Å wide columnar solvent channel, which is repeated throughout the crystal in a honeycomb-like pattern (Fig. 3a). The walls between these channels are formed by overlapping layers of protein molecules and are not penetrated by any larger channels.
(Fig. 3b), though smaller gaps exist (width up to \(-10\) Å), which would allow passage of solvent but not large molecules. Honeycomb patterns are recurrent in nature and in various types of nanomaterials. Indeed, it has been suggested that honeycomb-like protein crystals may be used for templating of nanomaterials or be developed into reaction vessels e.g. through coupling of functional compounds to engineered cysteines exposed to the wide columnar solvent channels\(^7\). Such applications may therefore also be envisaged for BAP29 vDED. The tetrameric crystal form also has a high solvent content (\(-74\%\)), but the channels are not as uniformly wide as in the dimeric form (supplementary fig. 2). It is however noteworthy that the two cysteines that do not engage in disulfide bonding (C171 of chains C and D), are fully exposed to the solvent channels, indicating that it is in principle possible to design disulfide cross-linked crystals, which expose free/reactive cysteines to the solvent.

**Conclusions**

Two structures have been determined of BAP29 vDED, which show that the domain can assemble into both a parallel dimer and an unexpected antiparallel tetramer of unclear physiological relevance. These structures strongly suggest that the previously noted pH sensitivity of folding is governed by close proximity of glutamates in the folded state. Furthermore, the crystal packing of the dimeric form exhibits two interesting features of potential bio- and nanotechnological relevance: The crystal is fully penetrated by 75x90 Å wide solvent channels arranged in a honeycomb-like pattern, and most remarkably, the single cysteine found in the domain forms a disulfide bond across a crystal contact with close to ideal geometry, resulting in complete cross-linking along the c-axis. Interestingly, disulfide cross-links can also be induced to form in the tetramer crystals, but these could not be as well characterized and are probably less attractive as a starting point for development of disulfide polymerized crystals. This may be the first report of a disulfide polymerized protein crystal.

**Notes and references**

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\(^3\) Electronic Supplementary Information (ESI) available: Supplementary Methods, table 1 and fig. 1-2. See DOI: 10.1039/b000000x/