Supplementary Material

Towards a high throughput system for high pressure cooling of cryoprotectant-free biological crystals

Authors

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1. High pressure cooling toolkit

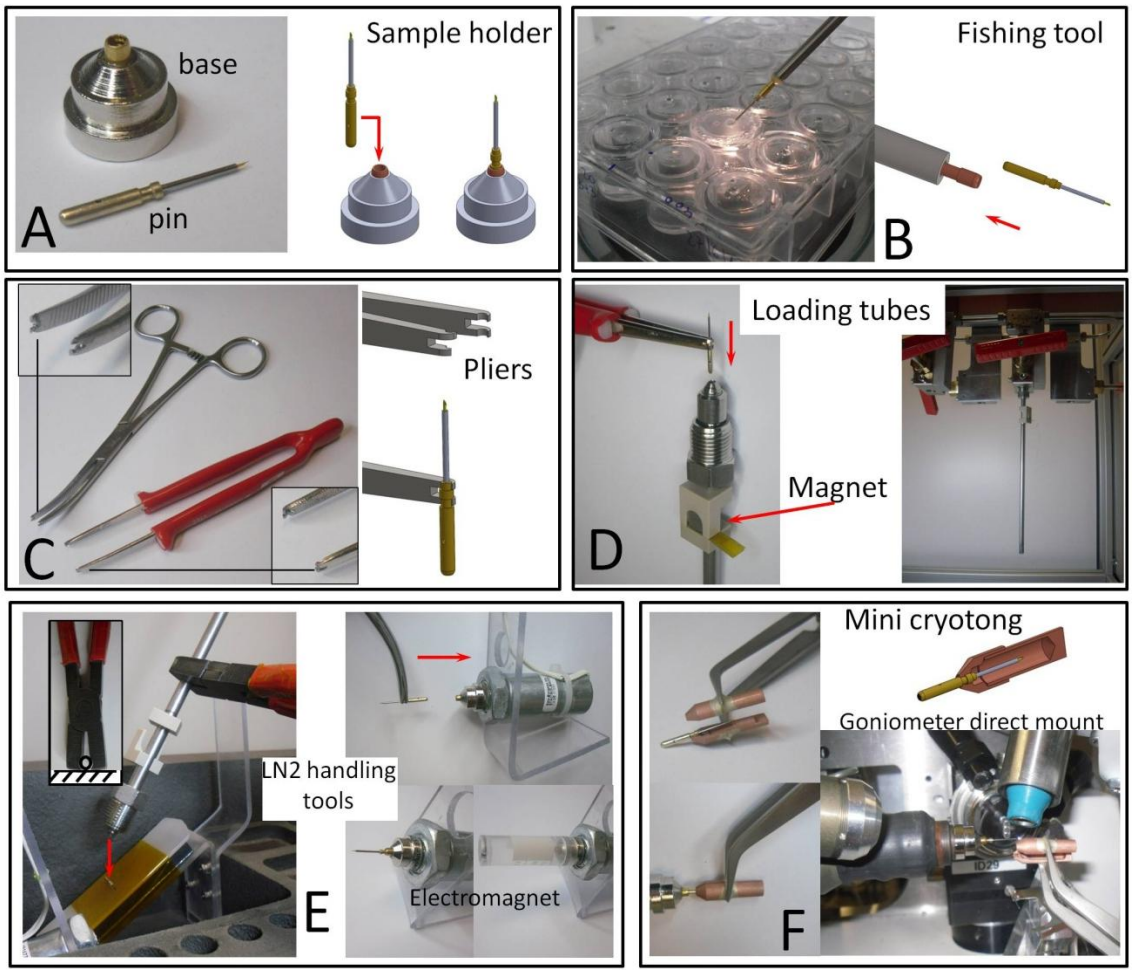

Figure sup-1: The cryo-toolkit. **A** cryopin. **B** fishing-pen. **C** cryo-pliers and tweezers. D Loading tubes operation. **E** Cryogenic handling. **F** Mini-cryotong.

Specific tools were designed to assist with each step of the high pressure cooling process; the complete toolkit is presented in the panel of images Fig. sup-1. For the crystal fishing operation, the sample holder pins (Fig. sup-1A) are placed in a fishing-pen (Fig. sup-1B) which allows a gain in stability and precision for catching crystals in drops. Cryo-compatible tweezers and pliers conceived with a slotted shape allow a firm grip on the shoulder of the pins which are too small for manual handling (Fig. sup-1C) when loading into the drop-tubes. The pins are held in place at the top part of the tubes by a magnet locked up in a clamp (Fig. sup-1D). After completion of the high pressure cooling operation, the pins are extracted from the tubes by turning them upside down with a cryo-claw and caught on a magnetic inclined plane (Fig. sup-1E). The pins are picked up in the liquid nitrogen bath with the tweezers, reconnected to their bases, covered by vials *via* an electromagnetic wand (Fig. sup-1E), and then loaded in a sample changer basket. As an alternate option, the pins can be manually transferred from the liquid nitrogen bath to the diffractometer axis inside a mini-cryotong (Fig. sup-1F). A smart operation of these tools allows transferring the four samples of the cooling cycle into the basket in less than 5 minutes.

2. Drop tubes geometry and temperature

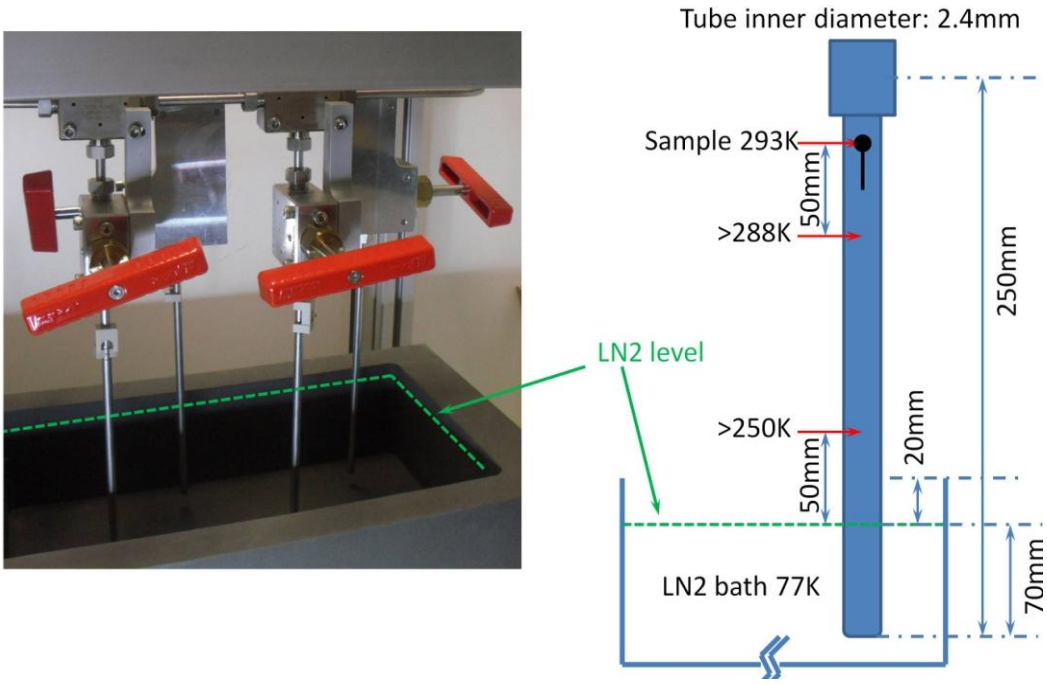

Figure sup-2: Drop tube geometry and temperature

We observed that the temperature gradient in the tube is established mostly in the static cold gas column in between the liquid nitrogen surface and the top of the Dewar. As consequence, this distance should be minimised; about 20mm in our case. We verified that the temperature of the tube at

50 mm above the liquid level does not fall below 250K and at 50mm from the sample does not fall below 288K (see Fig. sup-2). The drop tube cooling rate when plunging in LN2 has been measured placing a Pt-100 thermometer in the cold zone where the samples are cooled. This measurement aimed to compare the cooling rate of a connector closing the tube (as for the S.M. Gruner's apparatus) with that of a butt welded tube (our choice). The latter cooled down three times faster owing to a reduced thermal mass (Fig. sup-3). Taking that process time into account in the loading phase, afterwards the Helium gas still has to thermalise inside tube prior to cooling the samples, the choice of using a butt welded drop tube constitutes an improvement.

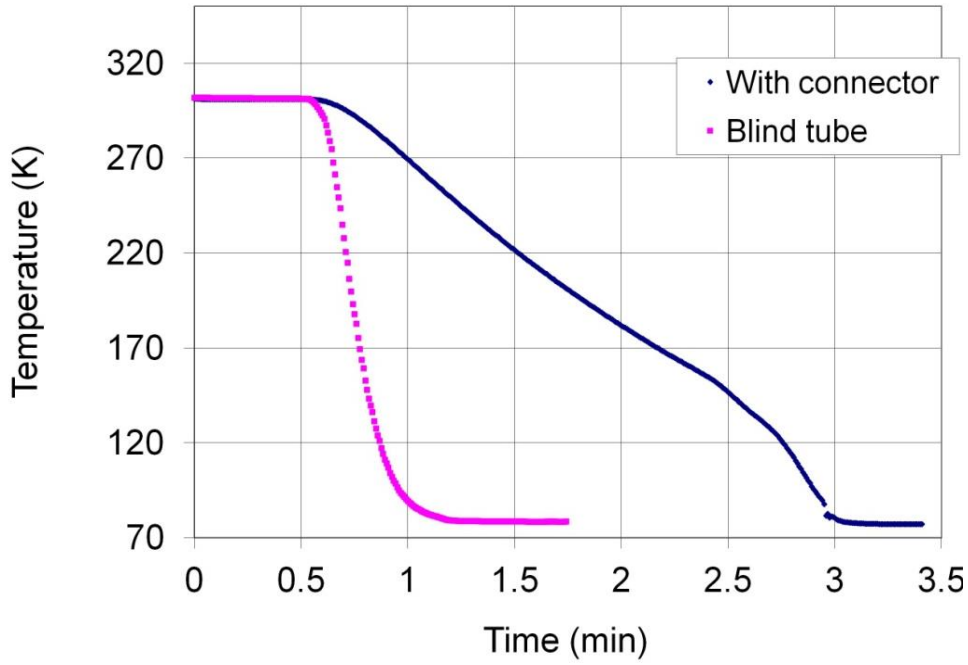

Figure sup-3: Drop tube cooling rate

The pressurisation step takes 5 minutes which gives the drop tubes sufficient time to cool down and samples to equilibrate in the Helium gas. Cooling the samples only takes a few seconds, and depressurisation takes a few more minutes. The cryo-toolkit allows mounting and unmounting the samples in less than 5 minutes each. Therefore, the full high pressure cooling cycle of 4 samples is safely and surely carried out within 20 minutes. Moreover, during a high pressure cooling session, 4 sets of 4 tubes are prepared to run continuously several cycles of the machine without having to additionally integrate their warming up and drying time.

3. Sample holder design

A female connector (Souriau, product RC16SE4K) is glued into the base of the sample holder (see Fig. sup-4). A stainless steel MicroTube™ (Hampton Research, Aliso Viejo, CA, USA, product HR4-923) is glued into the male connector to adjust the total holder length to 22mm (Spine standard).

A short Kapton® capillary at the pin extremity (length 2mm, diameter 100-300µm, wall thickness 25µm) is sufficiently X-ray transparent and mechanically rigid in the cryo-stream to ensure good diffraction data statistics. The spoon shape of the capillary facilitates sample fishing and handling, and maintains the sample hydration during 1-2 minutes in order to eliminate the use of non-penetrating cryoprotectant. The support presented sample holder (see Fig. sup-4) is Spine standard, but the system is highly versatile; the pins can be easily transferred in LN2 to different cap systems providing that they are equipped with the mating female connectors.

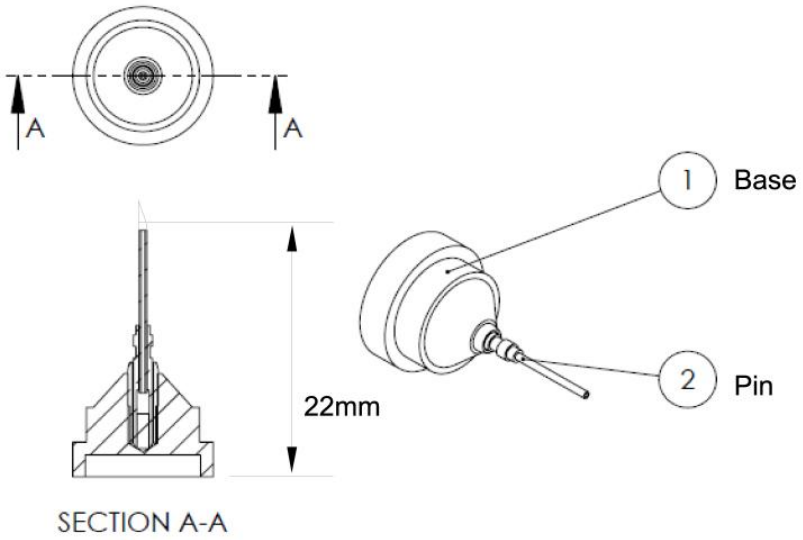

Figure sup-4: General drawing of the pin-base connectable system

4. High pressure system

The biological samples together with a drop of mother liquor are fished from crystallisation trays (Fig. sup-1B, Fig. 4-A) using the pins described in the main manuscript section 2.1 and supplementary section 3 without adding any cryoprotective agent to the mother liquor. They are subsequently loaded in the upper parts of the pressurising tubes at ambient pressure and temperature, fixed through the stainless steel tube walls by an external magnet. The tubes are then bolted to the machine and immediately pressurised to 10 MPa to stop dehydration of the crystals. Thereafter, the lower parts of the pressurising tubes are immerged into a LN2 bath. The pins, maintained at 294 K until reaching the equilibrium pressure of 200 MPa, are released by removal of the magnets and drop down into the cold zone of the tubes where the mother liquor is flash-transformed under high pressure into a HDA ice matrix embedding and protecting the crystals. Upon pressure release, the HDA phase

remains stable as long as the temperature is maintained below 120 K. Above this temperature, the HDA/LDA transition of the solvent would blow up the crystals.

The Fig sup-5 provides the details of the high pressure cooling apparatus with a two stage He-compressor (NovaSwiss, Cesson, France), a pressurisation bench, and a noble-gas sub-system with its cryo-trap. With this setup, approximately 2000 samples can be cryo-cooled using a standard He cylinder. The pressurisation bench also offers the option to switch the connection of the sample tubes to a Xenon (or a Krypton) gas cylinder at 3.6 MPa to prepare noble-gas derivatives prior to cooling the crystals.

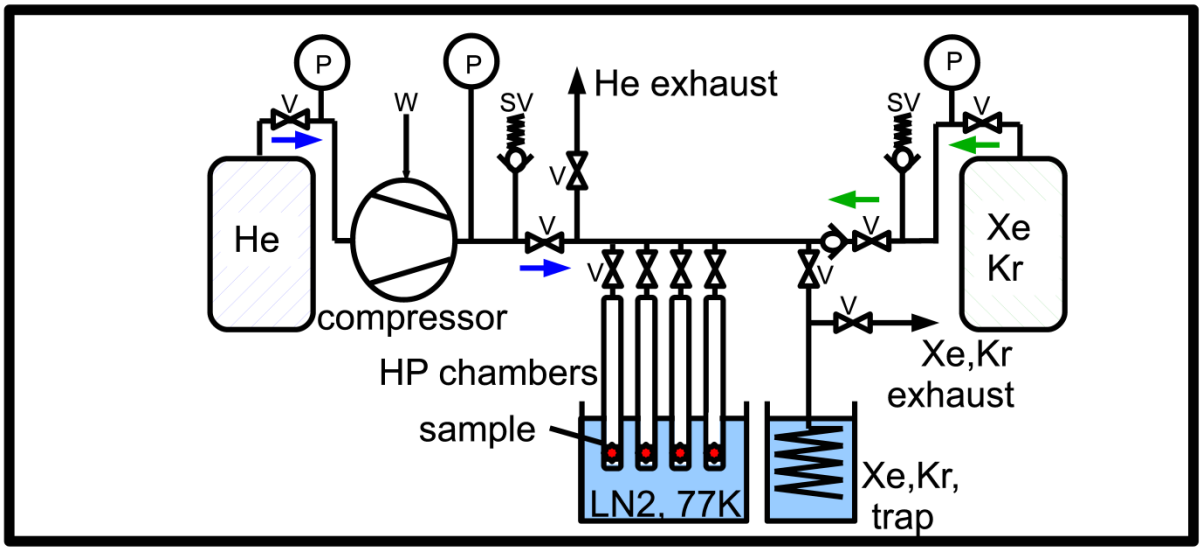

Figure sup-5: Scheme detailing the different subcomponents of the apparatus and the tubing connection (P= gauge, V= valve, SV= safety valve, W= external power).

5. Structural changes of some test samples

The high pressure cooling system has been tested with cryoprotectant free biological crystals from thermolysin, proteinase-K, insulin, lysozyme, FAE and thaumatin (preparations described in section 2.4 and table 1). The most representative results of pressure induced structural modifications are presented in section 3.2. We demonstrate that structural changes are few, local and generally concern flexible loops at the protein surface. Conclusively, HP-cooled structures are relatively isomorphous to AP-cooled structures. Here, in Fig. sup6 to sup9, we provide the HP-induced structural changes in 3D representation for the remaining proteins not presented in the main manuscript.

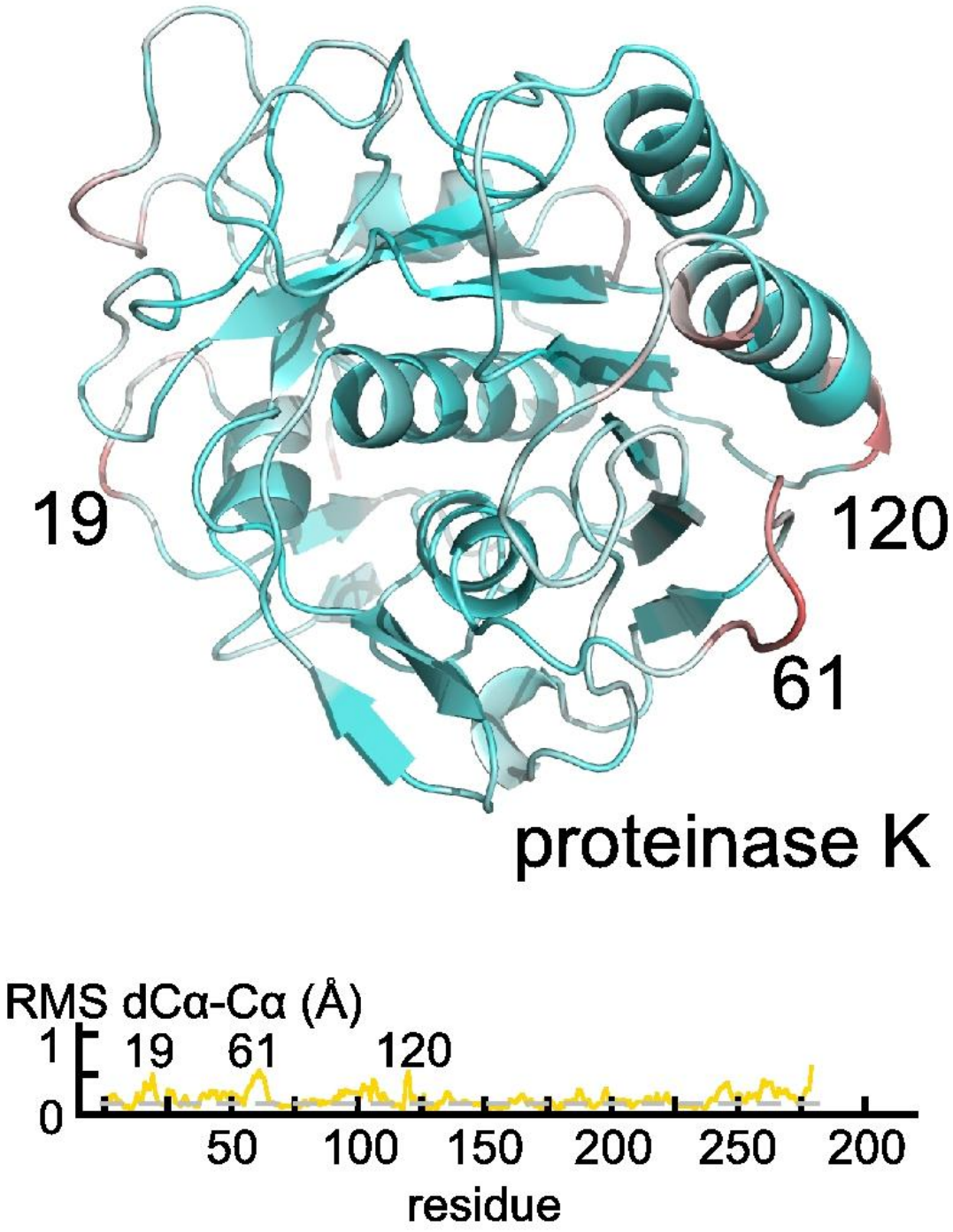

Figure sup-6: Detailed changes in proteinase K. Cα backbone displacements induced by pressure, 3D ribbon representations of structural changes coloured from cyan to red with increasing backbone displacements. 3 loops, containing residues 19, 61 and 120 at the surface of the protein undergo the largest modifications under pressure.

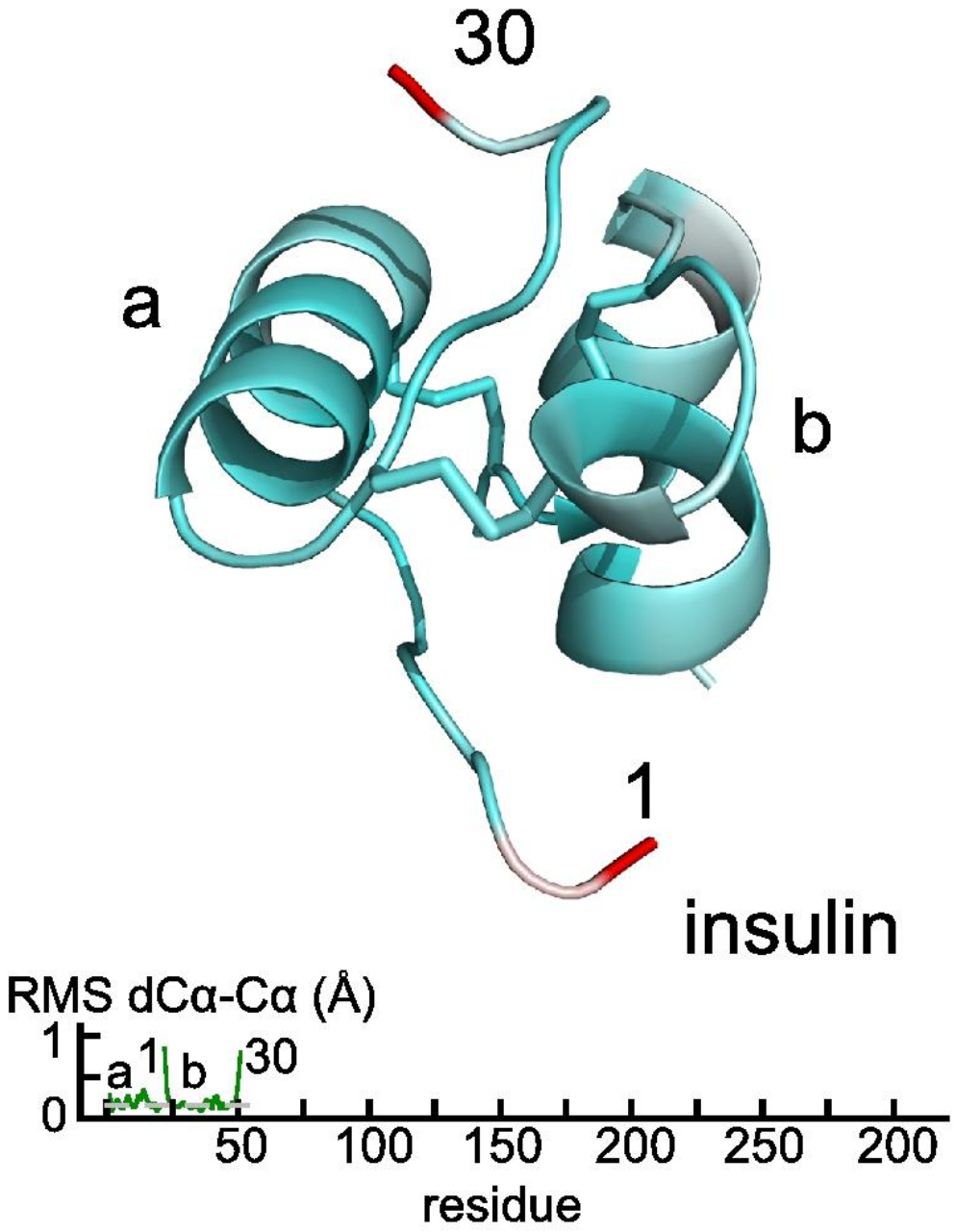

Figure sup-7: Detailed changes in Insulin. Cα backbone displacements induced by pressure, 3D ribbon representations of structural changes coloured from cyan to red with increasing backbone displacements. The N and C-ter extremities of domain b undergo large movement under pressure; this is an observation of reduced significance as those parts are in any case very flexible. Nevertheless, on the contrary, insulin which contains a high proportion of helices appears relatively non sensitive to pressure.

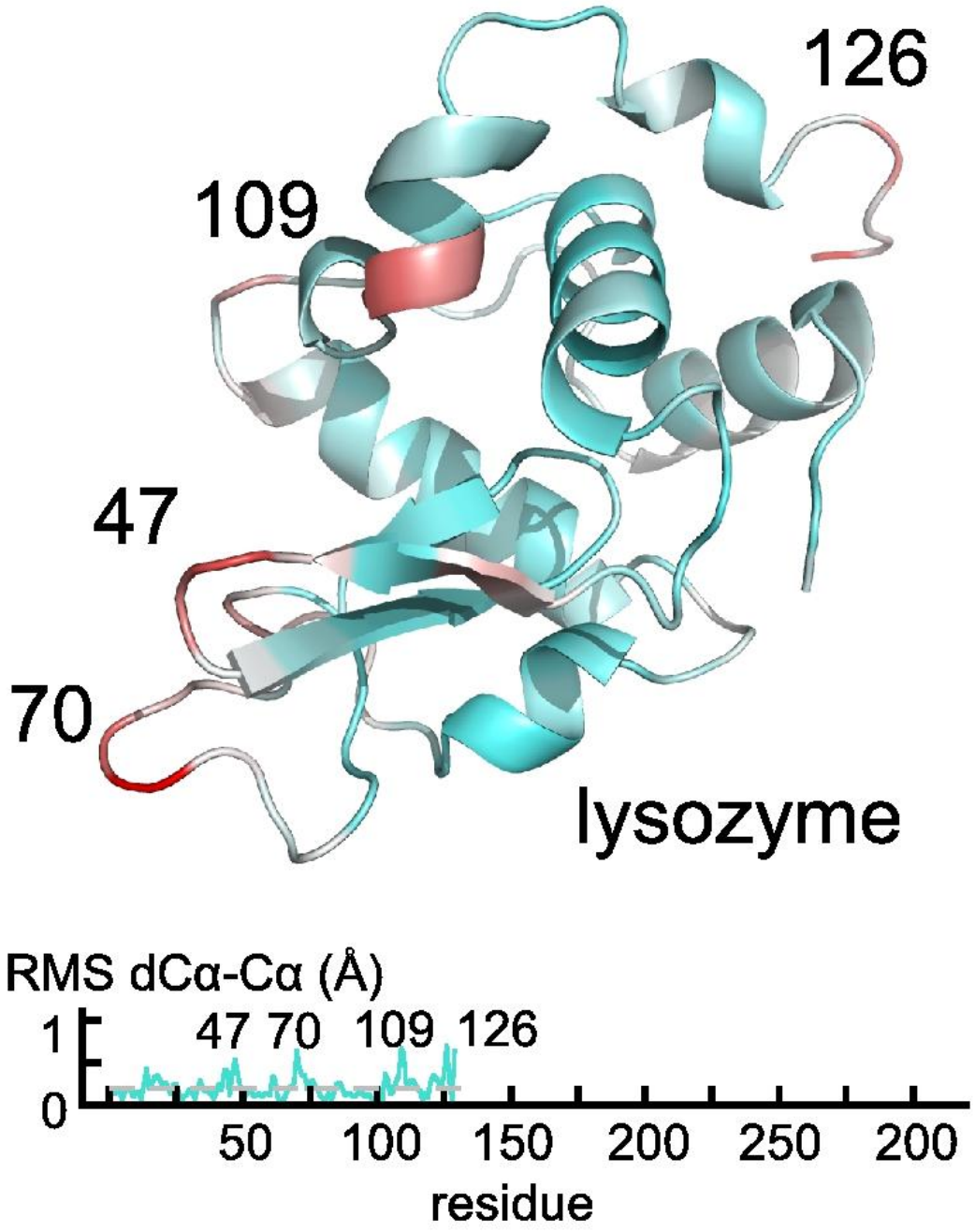

Figure sup-8: Detailed changes in lysozyme. Cα backbone displacements induced by pressure, 3D ribbon representations of structural changes coloured from cyan to red with increasing backbone displacements. 4 loops, containing residues 47, 70, 109 and 126 at the surface of the protein undergo the largest modifications under pressure.

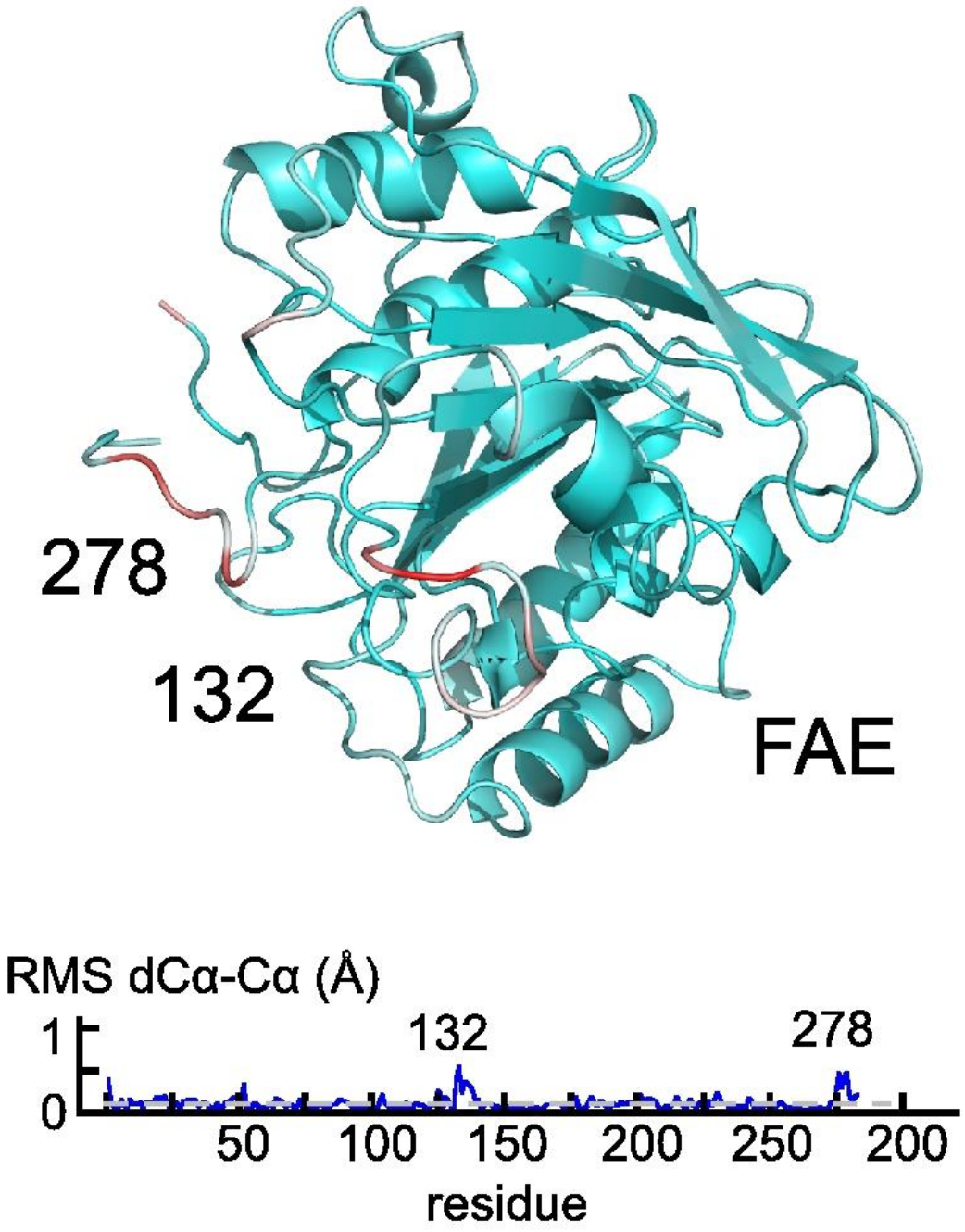

Figure sup-9: Detailed changes in FAE. Cα backbone displacements induced by pressure, 3D ribbon representations of structural changes coloured from cyan to red with increasing backbone displacements. The loop containing residue 132 reveals the highest displacement, this movement results in a pressure induced phase transition (see discussion in the manuscript). The C-ter extremity is the only other loop sensitive to pressure.