SUPPLEMENTARY INFORMATION

4D Cryo-Electron Microscopy of Proteins

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Material and Methods

Sample Preparation

Bovine insulin (Sigma) was dissolved into hydrochloric acid at a concentration of 10 mg/ml to give a final pH of 2.0. The solution was heated for 24 hours at 70°C and left at room temperature for seven days. The fibrils were purified by resuspending them after centrifugation into pure water. The fibrils were then treated with a tenfold molar excess of Congo red disodium salt (CALBIOCHEM) and vortexed to ensure good mixing. The unbound Congo red was subsequently washed off by five cycles of centrifugation (at 4000 rpm, 3220g). The final supernatant was colorless indicating that there was no unbound Congo red.

4D cryo-electron microscopy

Fibrils were applied to lacy carbon grids (Electron Microscopy Sciences) that were immediately plunge-frozen at liquid nitrogen temperature. 4D cryo-EM images were acquired in stroboscopic mode (Fig. 1, main text) using timed photoelectron packets (120
kV, LaB$_6$ source), a green pump pulse ($\lambda = 532$ nm, repetition rate 1kHz) and a Gatan 626 cryo-holder. Diffraction images were acquired at a CCD camera length of 0.75 m and 1.5 m. All analysis and rendering of figures was performed in MATLAB, Excel or Chimera$^2$.

**Calibration of Laser-Induced Temperature Jump**

Static electron diffraction patterns of the amyloid fibrils were acquired at room temperature (300 K) and in increasing 2 K increments to 306 K by precisely adjusting specimen heating using the temperature-controlled Gatan 626 cryo-holder (Fig. S2). By monitoring the change in radius of the 0.48 nm fibre diffraction rings as a function of temperature (Fig. S2), the thermal expansion coefficient, $\alpha$, of the insulin fibrils was determined from the gradient of the fitted straight line to be $1.9 \times 10^{-3}$ K$^{-1}$ in the range 300-306 K. Since the relative expansion of the fibril network at 300 K is $4.0 \pm 0.2 \times 10^{-3}$, the laser-induced temperature jump is ~ 2 K.

**Supporting References**


Supporting Figures

Figure S1. Close-up view of the amyloid fibril network (protein density shown in white) on a lacey carbon substrate (Scale bar, 200 nm).
Figure S2. Determination of the thermal expansion coefficient of insulin fibrils, $\alpha = 1.9 \times 10^{-3} \text{ K}^{-1}$, from highly stable, temperature-controlled static diffraction measurements.