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Supplemental information

Zinc determines dynamical properties and aggregation kinetics of human insulin

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Supporting Figures

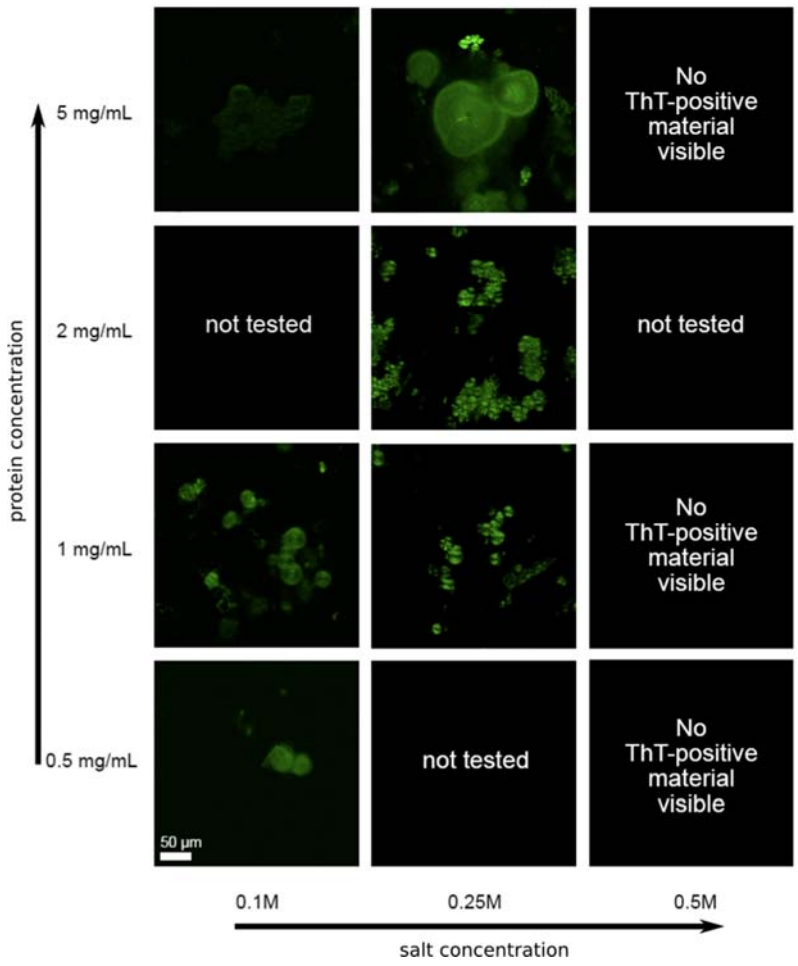

Figure S1: Increasing the salt concentration to 0.5 M prevents the formation of spherulites and fibrils
Human insulin was dissolved in the corresponding buffer and incubated at 60°C for 24h without shaking. The samples were then observed by optical and electron (data not shown) microscopy after addition of ThT. Protein concentration increases along the rows and NaCl concentration decreases down the columns.

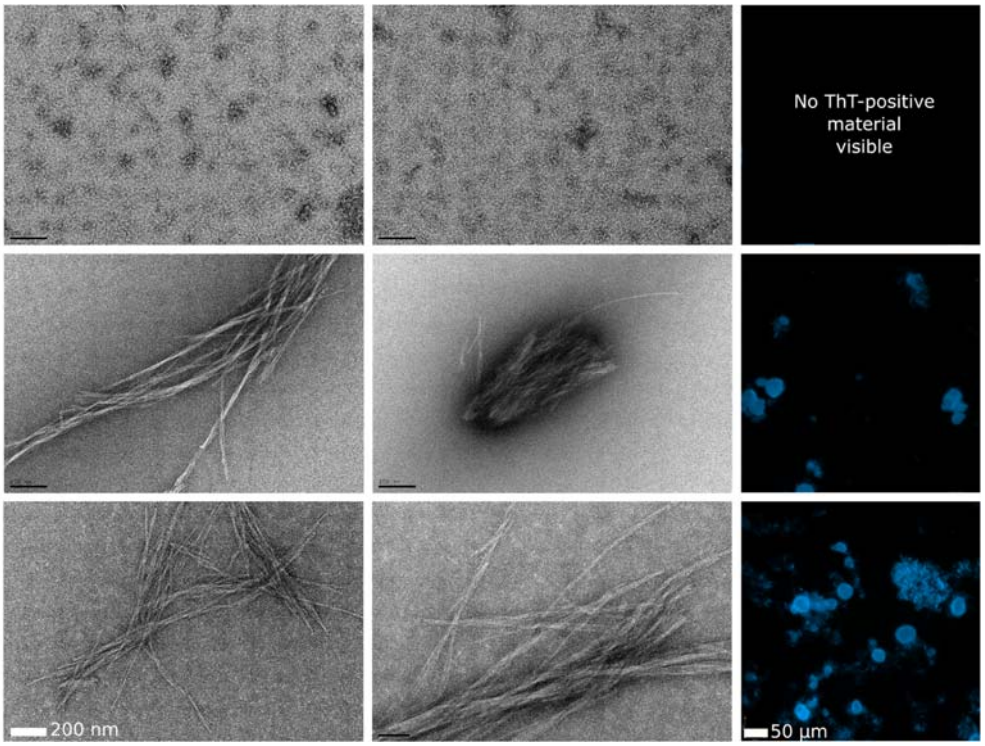

Figure S2: No aggregation is visible at 50°C without shaking but it is at higher temperatures.
Human insulin was dissolved in the corresponding buffer and incubated at 50, 60 or 70°C (upper, mid and lower row, respectively) for 24h without shaking. The samples were then observed by electron microscopy (left and middle) and by optical microscopy after addition of ThT (right). No amyloid aggregates were observed at 50° C, while both fibrils and spherulites were present at 60 and 70° C.

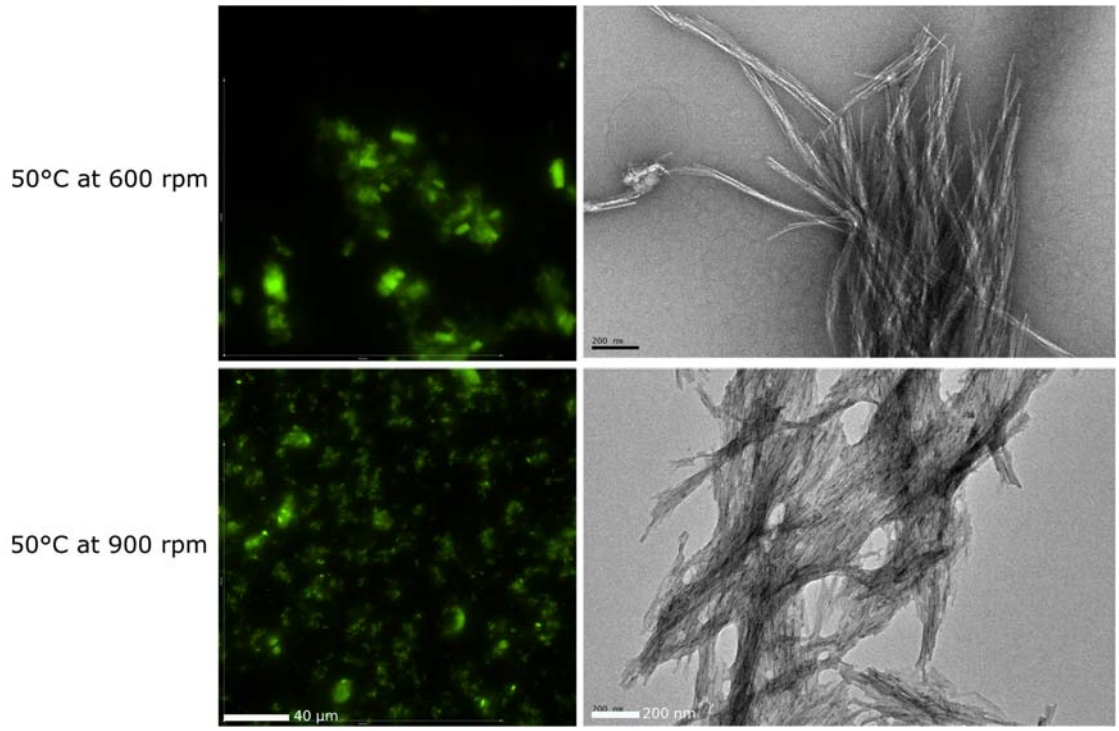

Figure S3: Shaking inhibits formation of spherulites but can affect the fibril morphology.
Human insulin was dissolved in the corresponding buffer and incubated at 50°C for 48h with the indicated shaking speed. The samples were then observed by optical microscopy after addition of ThT (left) and by electron microscopy (right).

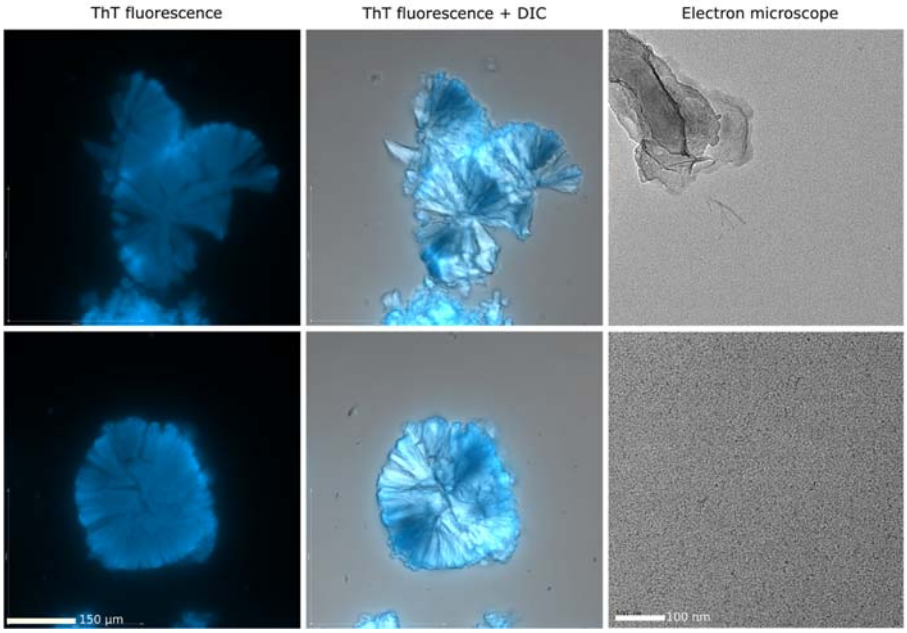

Figure S4: Pure spherulite sample can be obtained after centrifugation and lyophilization.
Human insulin was dissolved in the corresponding buffer and incubated at 60 or 70°C for 24 h without shaking. The samples were then observed by optical microscopy after addition of ThT (left - fluorescence only, and middle - differential interferential contrast (DIC) and fluorescence) and by electron microscopy (right). The two lines correspond to different locations on the glass slide.

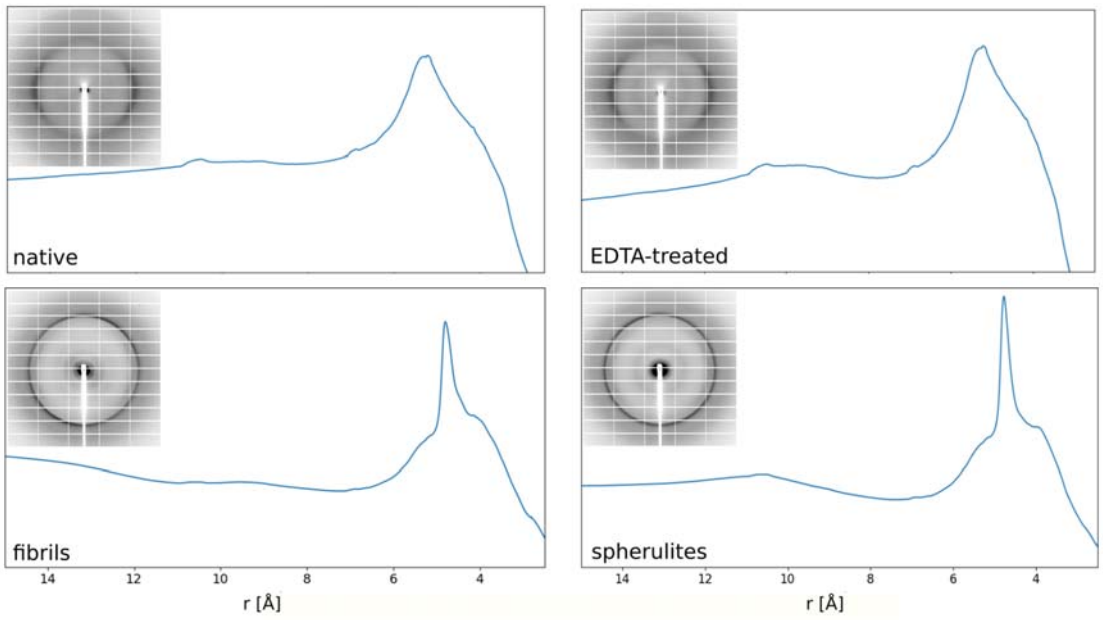

Figure S5: Aggregated insulin species present the cross-beta pattern.
A small fraction of the insulin powder sample that was used for neutron scattering was sealed in Mylar capillary for X-ray diffraction at the ID30B endstation of the ESRF. The 20 images collected were averaged for each sample, then the azimuthal integration was performed using pyFAI library to generate the lower panel plots. The narrow and intense peak at ~4.7 Å in both fibrils and spherulites is the signature of the presence of cross-β structures. The broad signal at ~5.2 Å corresponds to the scattering from the Mylar capillary. The insets show the diffraction pattern averaged over the 20 images.

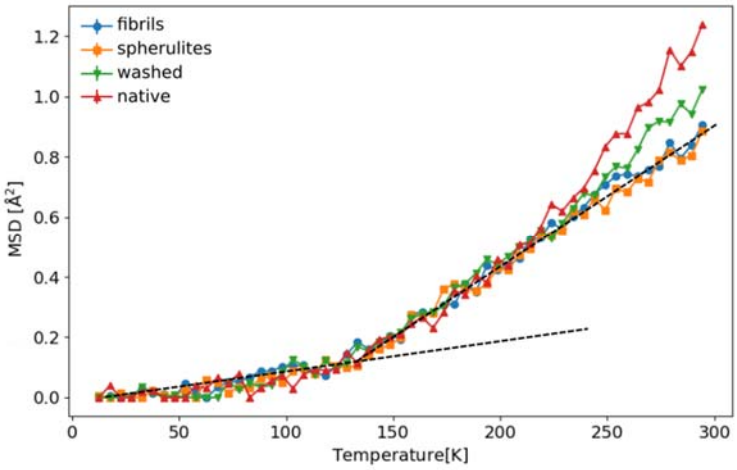

Figure S6: Only zinc affects insulin protein dynamics.
Mean square displacements (MSD), measured as a function of temperature by elastic incoherent neutron scattering with the backscattering spectrometer SPHERES (MLZ, Garching) for native insulin (red triangles), amyloid fibrils (blue circles) and spherulites (orange squares) and for washed insulin sample containing 0.12 zinc atom per insulin molecule (green triangles).

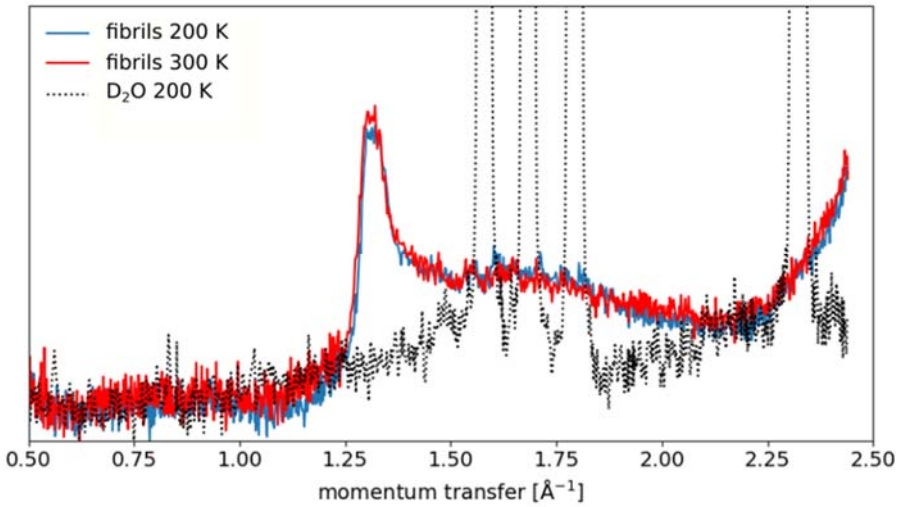

Figure S7: Insulin samples do not show any sign of crystalline ice.
The samples, contained in aluminum neutron cells, were mounted on the D16 instrument at the ILL. The diffraction patterns were recorded over an angle range between 12° and 112.5°, with a corresponding q-range of 0.05-2.5 $Å^{-1}$. Each scan was obtained by integrating the 2D signal over 20 minutes. Continuous lines refer to the fibril sample measured at 200 K (blue curve) and 300 K (red curve), dotted line to a sample of pure D_2O at 200 K.

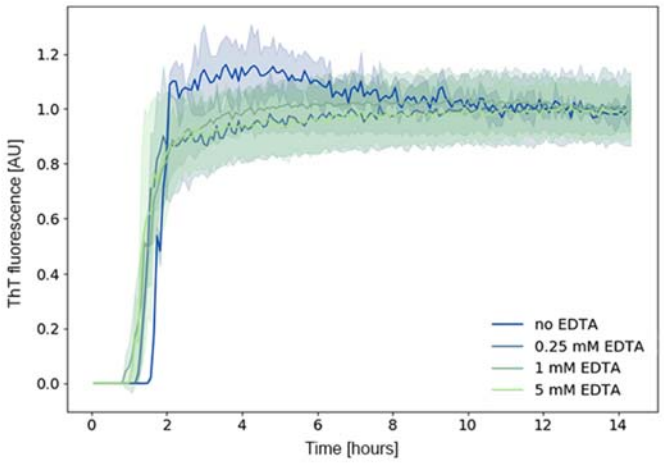

Figure S8: Insulin fibril formation under shaking is weakly affected by EDTA.
Insulin aggregation kinetics at 60° C with 300 rpm shaking as probed by Thioflavin T fluorescence as a function of EDTA concentration. The color shaded areas indicate the standard deviation for three independent aggregation processes.

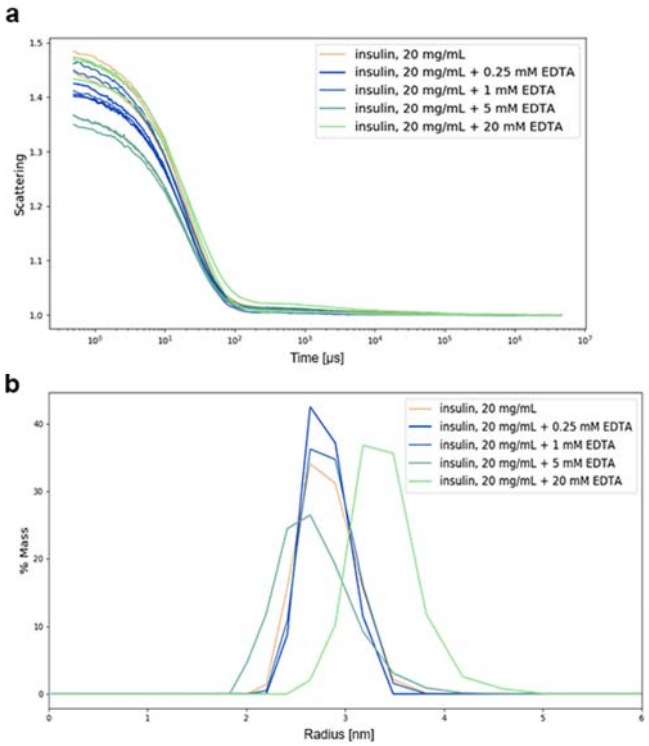

Figure S9: Removal of zinc barely affects insulin quaternary structure.
Insulin powder was dissolved in 0.25 M NaCl, pH 1.8 then an appropriate amount of filtered 0.26 M EDTA was added for each measurement of dynamic light scattering performed as described in methods. **a** Static scattering signal versus time for sample (measured three times, each consisting of 10 measurements of five seconds each). **b** Computed radius distribution of particles, only one peak is present with an area accounting for 99-100 % of the signal.

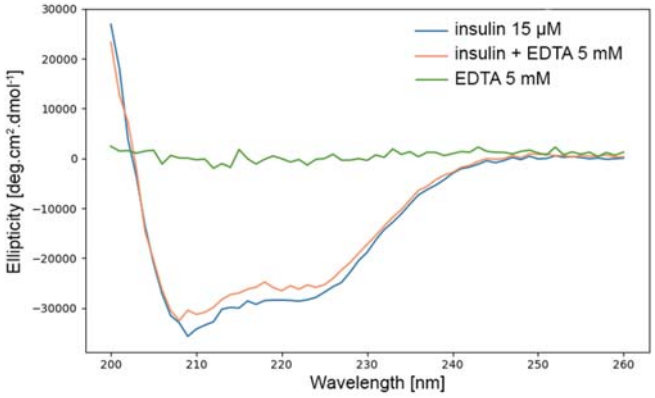

Figure S10: Zinc removal has no effect on insulin secondary structure content
Insulin was dissolved in pure water to a final concentration of 2 mg/mL by lowering the pH using sulfuric acid. It was then filtered and diluted again with or without EDTA to reach the indicated concentrations. Data were acquired at 20 nm/s with six accumulations on a JASCO spectrophotometer.

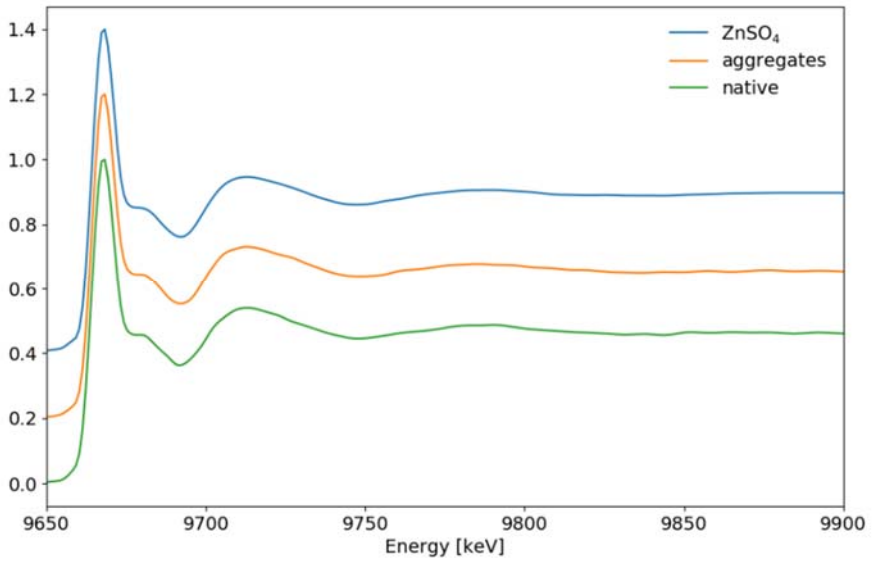

Figure S11: XANES spectra of $ZnSO_4$, native insulin and insulin aggregates
Zinc sulfate was dissolved in a 0.25 M NaCl, pH 1.8 solution. Similarly, the native insulin was dissolved in the same buffer, and the aggregates were formed by incubating half of the native insulin solution at 60°C for 24 hours. The XANES spectra were recorded as described in methods on the DUBBLE beamline at the ESRF in Grenoble, France.

Supporting Tables

	Sulfur		Zinc	
	No. of atoms per protein molecule	LOD†	No. of atoms per protein molecule	LOD†
Native	6*	0.05	1.56	0.03
	6*	0.07	1.46	0.07
Average		**0.06**	**1.50 (±0.17)**	**0.05**
Spherulites	6*	0.01	<0.001	0.001
	6*	0.01	<0.001	0.001
	6*	0.01	<0.001	0.001
Average		**0.01**	**<0.001 (±0.001)**	**0.001**
Washed	6*	0.01	0.101	0.004
	6*	0.01	0.145	0.003
	6*	0.01	0.101	0.003
Average		**0.01**	**0.116 (±0.032)**	**0.003**
EDTA treated	6*	0.013	0.020	0.003
	6*	0.012	0.020	0.003
	6*	0.010	0.023	0.003
Average		**0.012**	**0.021 (±0.005)**	**0.003**
Fibrils	6*	0.01	<0.001	0.001
	6*	0.01	<0.001	0.001
Average		**0.01**	**<0.001 (±0.001)**	**0.001**

* Assumed values from protein sequence
† LOD = Limit of detection (atoms/protein molecule)

Table S1: Zinc is hardly detected in aggregated samples
Measurement on the µPIXE beamline was performed as described in the text. The table reports the values obtained for the individual measurements carried out on each sample, as well as the averages.

	Native insulin	Aggregated insulin	$ZnSO_4$ solution
N_1	6 ± 0.5	6	6
R_1 [Å]	2.091 ± 0.005	2.083 ± 0.005	2.091 ± 0.005
σ^2_1 [Å^2]	(5 ± 0.5) e-3	(3.9 ± 0.7) e-3	(4 ± 0.5) e-3
ϑ_1 [deg]	150 ± 10 (R_2 = 4.042; σ^2_2 = 1e-2)	-	-
ϑ_2 [deg]	147 ± 5 (R_2 = 4.40; σ^2_2 = 9.7e-3)	-	-

Table S2: Parameters of EXAFS data analysis