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Ab initio phasing macromolecular structures using electron-counted MicroED data

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Structures of two globular proteins were determined ab initio using microcrystal electron diffraction (MicroED) data that were collected on a direct electron detector in counting mode. Microcrystals were identified using a scanning electron microscope (SEM) and thinned with a focused ion beam (FIB) to produce crystalline lamellae of ideal thickness. Continuous-rotation data were collected using an ultra-low exposure rate to enable electron counting in diffraction. For the first sample, triclinic lysozyme extending to a resolution of 0.87 Å, an ideal helical fragment of only three alanine residues provided initial phases. These phases were improved using density modification, allowing the entire atomic structure to be built automatically. A similar approach was successful on a second macromolecular sample, proteinase K, which is much larger and diffracted to a resolution of 1.5 Å. These results demonstrate that macromolecules can be determined to sub-Ångström resolution by MicroED and that ab initio phasing can be successfully applied to counting data.
Results
Preparing lysozyme crystals for MicroED experiments. Crystals of triclinic lysozyme were grown in batch (Methods). The crystals were visible under a light microscope and were initially about 10 µm in size. A slurry of these microcrystals was vitrified on electron microscopy grids. Grids were loaded into a dual-beam FIB–SEM where individual crystals that were at least 5 µm from a grid bar and at least three grid squares away from the edge of the grid were identified. The crystals were then coated in multiple layers of platinum for protection from the ion beam during the milling process. Each crystal was then milled into a thin lamella approximately 300 nm in thickness, about the ideal thickness for MicroED data collection at an accelerating voltage of 300 kV based on prior mean free path measurements (Fig. 1a,b and Methods).

The milled lamellae were then loaded under cryogenic conditions into a Titan Krios TEM. Lamellae were identified by low-magnification montages in which each site appeared as a semi-transparent shape suspended above an empty strip. Eighteen of the 20 milled lamellae were found in the TEM; two did not survive the cryotransfer step. For each lamella, the eucentric height was

![Figure 1](image-url)
Collecting MicroED data in counting mode. MicroED data were collected in electron-counting mode using a Falcon 4 direct electron detector (Methods and Supplementary Fig. 1c,d). This camera allows for highly accurate detection of single electron events. However, the number of electrons that can be counted in each pixel of each frame is limited. To ensure accurate reporting of the intensities, the exposure rate must be kept very low. This strategy reduces errors caused by too many electrons hitting the same pixel within a readout cycle of the detector but risks missing weak reflections in the background. These stringent requirements were met by greatly reducing the exposure rate and compensating by increasing the total exposure time (Methods). This strategy prevents the strong reflections from overwhelming or damaging the detector while weak high-resolution reflections are sampled at sufficient frequency to recover their intensities (Fig. 1d and Supplementary Fig. 1).

Multiple settings had to be adjusted to achieve a suitably low exposure rate for these experiments. Importantly, the camera’s dose protector, which automatically retracts the camera when the microscope enters diffraction mode, must be disabled (Methods). The smallest second condenser lens aperture (C2) was coupled with the highest spot size possible on our instrument (50 µm and spot size 11). The instrument was kept in microprobe mode to avoid an approximately fivefold increase in exposure rate that occurs by deactivating the condenser mini lens. Because these experiments were conducted on a Titan Krios, the beam size could be changed while maintaining a near-perfect parallel illumination. The beam diameter was spread to 25 µm to further reduce the exposure per unit area. Together, these modifications reduced the total exposure per area by a factor of up to 10 compared to prior experiments on this instrument\(^1\). Further, the data were collected as a 420-s exposure up to 4 × 420 s. The converted frames were processed using standard crystallographic software\(^{26,27}\). The space group for all 18 lysozyme crystals was determined to be P1, and the unit cell was determined to be (a, b, c) = (26.4±0.15 Å, 30.72±0.30 Å, 33.01±0.21 Å), (α, β, γ) = (88.32±0.25°, 109.09±0.38°, 112.07±0.32°) (Table 1). These stringent requirements were met by greatly reducing the exposure rate and compensating by increasing the total exposure time (Methods). This strategy prevents the strong reflections from overwhelming or damaging the detector while weak high-resolution reflections are sampled at sufficient frequency to recover their intensities (Fig. 1d and Supplementary Fig. 1).

Solving lysozyme at subatomic resolution. The movies from the Falcon 4 were sliced into either 1.0-s or 0.5-s segments, 420 or 840 individual frames, each 2,048 × 2,048 16-bit pixels in size, spanning a rotation of 0.075–0.2°. Images were converted to SMV format using the MicroED tools\(^3\) adapted for the Falcon 4–EPU-D–Velox metadata format. The total size of a compressed MicroED movie in counting mode for these exposures is typically 2.2 GiB (up to 14,455 frames during 420 s). The converted frames were processed using standard crystallographic software\(^{26,27}\). The space group for all 18 lysozyme MicroED datasets was found to be P1, and the unit cell was determined to be (a, b, c) = (26.4±0.15 Å, 30.72±0.30 Å, 33.01±0.21 Å), (α, β, γ) = (88.32±0.25°, 109.09±0.38°, 112.07±0.32°) (Table 1). The data were merged to increase completeness. The subsequent merging steps identified two lysozyme lamellae that correlated poorly with the other 16 integrated datasets. These two datasets were discarded. A high-resolution cutoff at 0.87 Å was applied, corresponding to 0.9011–0.87 Å. To ensure accurate reporting of the intensities, the exposure rate must be kept very low. This strategy reduces errors caused by too many electrons hitting the same pixel within a readout cycle of the detector but risks missing weak reflections in the background. These stringent requirements were met by greatly reducing the exposure rate and compensating by increasing the total exposure time (Methods). This strategy prevents the strong reflections from overwhelming or damaging the detector while weak high-resolution reflections are sampled at sufficient frequency to recover their intensities (Fig. 1d and Supplementary Fig. 1).

### Table 1 | MicroED crystallographic table of triclinic lysozyme

| MicroED structure of triclinic lysozyme | EMD 25184 |
|----------------------------------------|-----------|
| Accelerating voltage (kV)             | 300       |
| Electron exposure (e− Å⁻²)            | 0.64      |
| Wavelength (Å)                        | 0.0197    |
| No. crystals                          | 16        |
| Resolution range (Å)                  | 16.05–0.87 (0.9011–0.87) |
| Space group                           | P1        |
| Unit cell (a, b, c) (Å)               | 26.4±0.15, 30.72±0.30, 33.01±0.21 |
| (α, β, γ) (°)                         | 88.32±0.25, 109.09±0.38, 112.07±0.32 |
| Total reflections (no.)               | 569,407 (5,797) |
| Unique reflections (no.)              | 64,986 (2,783) |
| Multiplicity                          | 8.8 (2.1) |
| Completeness (%)                      | 87.55 (37.64) |
| l/σ                                  | 6.23 (0.66) |
| Wilson B factor                       | 9.44      |
| Reflections used for Rwork (no.)      | 64,955 (2,783) |
| Reflections used for Rfree (no.)      | 3,165 (128) |
| Rwork                                | 0.1969    |
| Rfree                                 | 0.2214    |
| No. non-hydrogen atoms                | 1,190     |
| Macromolecules                        | 1,018     |
| Ligands                               | 16        |
| Solvent                               | 156       |
| Protein residues (no.)                | 129       |
| Protein residues-μS                  | 0.027     |
| Protein residues-σ                 | 2.2       |
| Ramachandran favored (%)             | 98.43     |
| Ramachandran allowed (%)             | 1.57      |
| Ramachandran outliers (%)            | 0         |
| Rotamer outliers (%)                 | 0.93      |
| Clashscore                            | 5.44      |
| Average B factor                     | 14.39     |
| Macromolecules                        | 10.93     |
| Ligands                               | 16.51     |
| Solvent                               | 36.77     |

EMD, Electron Microscopy Data Bank; PDB, Protein Data Bank. Values in parentheses in column 2 denote the highest resolution shell.
to the point where the CC_{1/2}, the correlation coefficient between randomly chosen half-datasets, was still significant at the 0.1% level. The overall completeness of the final dataset was 87.5%, with all resolution shells below the resolution of 1 Å being >95% complete (Table 1, Supplementary Table 1 and Supplementary Fig. 3).

Phasing was performed by automatically placing an idealized helical triple-alanine fragment followed by density modification (Fig. 1e and Methods). A single helical fragment of only three alanine residues was sufficient to determine the entire lysozyme structure. After automatic placement in Phaser, an interpretable map was produced following 144 rounds of dynamic density modification, resulting in a map showing individually resolved atoms throughout the entire unit cell where all residues and several NO₃⁻ ions could be unambiguously identified (Fig. 2 and Supplementary Videos 1 and 2). The density-modified map ($E, \varphi$) was similar to the final $(2mF_o - DF_c, \varphi)$ map after refinement. A complete model was automatically built into the density-modified map given only the sequence and without consulting their known structures (Fig. 2). For this structure, two C-terminal residues and several solvent-exposed side chains were either partially or entirely absent in the map, even after final refinement. They were also poorly resolved in X-ray investigations of triclinic lysozyme at similar resolutions (Supplementary Fig. 4). The final model was refined using electron scattering factors.

Determining proteinase K structure at near-atomic resolution. The results from lysozyme using electron-counted MicroED data are very promising. However, it is not entirely...
surprising that sub-Ångström data could be determined ab initio even considering the larger size of lysozyme compared to that of small molecules or peptides. To test if other electron-counted MicroED data would perform similarly, this approach was tested again using a sample of the serine protease proteinase K, which is much larger and more representative of the average size of globular proteins in the cell. The crystals were grown in batch, and sample preparation was very similar to the approach used for the triclinic lysozyme microcrystals. The crystals were identically milled and screened (Supplementary Fig. 2). Four proteinase K crystals were found on a single grid that met the selection criteria. MicroED data were collected with an overall similar approach using electron counting (Methods). We similarly found that essentially all pixel values fell within the linear range of the detector and were well below the threshold for damaging the detector (Supplementary Fig. 1 and Methods).

The data were indexed, integrated and scaled using the same software as that used for the triclinic lysozyme data. The Laue class was determined to be 4/mm with a unit cell of \((a = b, c) = (67.08 \pm 0.21 \text{ Å}, 106.78 \pm 0.36 \text{ Å}), (a = b = c) = (90 \pm 0^\circ)\). Two of the four proteinase K lamellae integrated with lower internal consistency and were therefore discarded. A high-resolution cutoff was applied using the same criteria at 1.5 Å, and the overall completeness of the final dataset was 98.8% (Supplementary Tables 2 and 3 and Supplementary Fig. 3). The overall statistics for these merged crystals were better than those from any prior investigations on the same microscope using a CMOS detector.

Phasing larger proteins below atomic resolution is more challenging. The same approach was used as that for lysozyme for phasing, namely, automatically placing idealized helices and then improving the solution using density modification. Four 14-residue-long ideal alanine fragments were automatically placed using Phaser. This solution was extended using multiple rounds of chain tracing and density modification, similar to the procedure implemented by ARBIMBOLDO_LITE. The correct space group was determined by attempting initial fragment placement in all possible space groups for this point group and then tracing the best solutions. The solution in space group P4_2_2 yielded a model in which nearly the entire protein backbone was successfully built. In the final round, this procedure traced 253 of 278 alanine residues (Supplementary Table 2 and Supplementary Fig. 6) and the map showed clear side chain densities. The model was completed automatically from the backbone trace given only the sequence. The structure was similarly refined using electron scattering factors.

Discussion

Ab initio phasing using electron-counted data was successful for both lysozyme and proteinase K following a similar approach. The lysozyme structure was determined from an idealized alanine helical fragment of only 15 atoms, corresponding to less than 1.5% of the structure. The maps calculated from only this small fragment showed density only around the placed atoms and uninterpretable noise elsewhere (Fig. 1c): not even the correct side chains for the three placed residues could be reasonably modeled at this stage. However, the entire protein structure along with solvent ions and water molecules were individually resolved after density modification (Fig. 2). The proteinase K structure was determined using four idealized helices, each made of fourteen alanine residues or 280 atoms (Methods and Supplementary Fig. 6), corresponding to about 13% of the structure. This solution also required density modification after placing the initial fragments. Its lower resolution and larger size meant that this structure required multiple rounds of chain tracing and density modification but was also determined ab initio using electron-counting data. This procedure has previously been attempted several times on non-counting datasets without success. The improved data quality from the electron-counted data was critical to ab initio phasing macromolecular crystals using MicroED data.

This study demonstrates that MicroED data can be collected using a Falcon 4 direct electron detector in counting mode. Previous attempts at collecting MicroED data using counting on a Falcon 3 direct electron detector failed because the Falcon 3 detector is slower (40 Hz) and has a much smaller linear range of only about 1 electron per pixel per second. Therefore, the Falcon 3 was previously used in integrating (or linear) mode in which it operates similarly to other CMOS-based detectors. In the current study, the Falcon 4 direct electron detector was used, which is capable of recording images at 250 Hz. Combining the faster frame rate with an ultra-low-dose spread over a long exposure meant that single electron events could be counted and the recorded intensities could be accurately integrated.

The data presented here were collected from crystal lamellae that were milled to an ideal thickness to match the mean free path of electrons accelerated in 300 kV. Milling crystals can either be used to make large crystals smaller or to remove material that embeds small crystals and prevents access for MicroED investigation, such as membrane proteins grown in viscous bicipelles or lipidic cubic phase. Regardless of the reason, milling crystals into an ideal thickness is a good practice and is recommended for extracting the most accurate data. The lysozyme crystals milled in the current study diffracted to sub-Ångström resolutions even after being milled by a gallium ion beam. This suggests that milling crystals may preserve the diffractive power of the sample. Indeed, the viability of collecting sub-Ångström data from crystalline lamellae further solidifies ion beam milling as the preferred approach for preparing macromolecular crystals for MicroED experiments, as the crystals and surrounding material can be shaped and thinned to ideal thicknesses for any accelerating voltage. Importantly, at a mere 300 nm, these lamellae matched the inelastic mean free path of a 300-keV electron. This reduces the contributions of inelastic scattering and multiple scattering events, likely contributing to greater accuracy in the data.

The results presented here open the door for future investigations of phasing macromolecular protein crystals at or beyond near-atomic resolutions using MicroED data. Attempting to apply direct methods as implemented in, for example, Shake-Bake or SHELXL is an exciting avenue for future investigations because not all proteins will have helices to place for the starting phases. Further developments in ab initio phasing of MicroED data could be used to both automate the process and extend the necessary resolution to cover more challenging structures, such as membrane proteins that do not routinely reach resolutions better than about 3 Å. Regardless, the ability to probe membrane protein structure by MicroED is advantageous as electrons can probe the charge properties in the sample. The quality of MicroED data obtained by counting would likely be further improved with the addition of an energy filter as this made large differences in the quality previously obtained on integrating detectors and this detector is compatible with an energy filter from the same manufacturer. Given the importance of phase improvement in traditional X-ray crystallography, the successful application of density-modification algorithms compatible with MicroED data will be of critical importance for solving structures without known homologs or very difficult structures at lower resolutions.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-022-01485-4.
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Methods

Materials. Hen egg white lysozyme (*Gallus gallus*) and protease K (*Engyodontium album*) were purchased from Sigma-Aldrich and used without further purification. Sodium acetate, pH 4.5, MES–NaOH, pH 6.5, calcium chloride and sodium nitrate stock solutions were used directly from Hampton crystallization kits and diluted using Milli-Q water as needed. The magic triangle kit was purchased from Hampton.

Crystallization of triclinic lysozyme. Crystals were prepared similarly to the protocol originally detailed by Legrand et al. and then subsequently described by Heijna et al.. Hen egg white lysozyme was dissolved in 0.2 M NaNO₃, 0.05 M sodium acetate, pH 4.5, to a concentration of 10 mg/ml. A total volume of approximately 0.5 ml was prepared in a cold room and vortexed at the maximum setting for approximately 1 min immediately after mixing. The tube containing this mixture was left at 4°C overnight. The next morning, the tube was observed to be nearly filled with crystals. The sample was removed from the cold room, sealed with parafilm and left on a benchtop at room temperature (approximately 23°C) for 1 week. After this time, very large, clear crystals accumulated on the bottom of the tube, and the remainder of the liquid appeared transparent. Small (1-1 μl) aliquots from the center of the liquid that appeared clear by eye were found to contain a slurry of small, irregularly shaped crystals when viewed under a light microscope.

Crystallization of protease K. Protease K was crystallized as described. Protein powder was dissolved at a concentration of 40 mg/ml in 20 mM MES–NaOH, pH 6.5. Crystals were formed by mixing a 1:1 ratio of protein solution and a precipitant solution composed of 0.5 M NaNO₃, 0.1 M CaCl₂, and 0.1 M MES–NaOH, pH 6.5, in a cold room at 4°C. Microcrystals grew overnight.

Grid preparation. Quantifoil Cu 200 R 2/2 holey carbon TEM grids were glow discharged for 30 s at 15 mA on the negative setting immediately before use. Grids were loaded into a Leica GP2 vitrification robot. The robot sample chamber was loaded with filter paper and set to 4°C and 95% humidity for 1 h before use. Protein crystals (3 μl) from the center of either the protease K or lysozyme tubes were applied to the carbon side of the glow-discharged grid and allowed to incubate for 30 s. Grids were then gently blotted from the back for 20 s. For lysozyme, the grids were then immediately plunged into super-cooled liquid ethane. For protease K, 3 μl 0.25 M ISC, 0.5 M NaNO₃, 0.1 M CaCl₂, 0.1 M MES–NaOH, pH 6.5 was added as described. The lysozyme grids were blotted more from the back for 20 s and then immediately plunged into liquid ethane. Vitrified grids were stored at liquid nitrogen temperature before further experiments.

Focused ion beam and scanning electron microscopy. The vitrified grids were inserted into autogrid clips at liquid nitrogen temperature. After clamping, the grids were loaded into a cryo-transfer shuttle and inserted into a Thermo Fisher Aquilos dual-beam FIB–SEM operating at liquid nitrogen temperatures. The samples were covered with first fine and then rough coats of sputtered platinum immediately after loading. An additional layer of protective platinum was added using the gas-injection system. Gas-injection system platinum coating was conducted in the mapping position with a working distance of 12 mm. In this way, a platinum layer approximately 1 μm thick was slowly deposited over 30 s and continually monitored using the electron beam.

Whole-grid montages of each grid were taken at low magnification using MAPS software (Thermo Fisher). Crystals on the vitrified grids were identified in the FIB–SEM such that each crystal was not within 5 μm of a grid bar, not within three grid squares of the edge and not within 25 μm of another selected crystal (Fig. 1a and Supplementary Fig. 2a). Twenty such crystals across two grids were prepared over 20 μl for lysozyme, and five protease K crystals on one grid were prepared in 1 μl. Identified crystals were brought to the eutectic position and inspected in both the electron and ion beams. Milling was conducted as described previously. Briefly, each crystal was roughly milled to an approximate thickness of 3 μm and an approximate width of 5–10 μm using an ion beam current of 300 pA and the standard rectangular milling patterns. Each crystal was then finely milled to a thickness of approximately 500 nm and a width of approximately 5–10 μm using an ion beam current of 100 pA. Finally, each lamella was polished using an ion beam current of 10 pA to a thickness of approximately 300 nm and a width of 3–8 μm using a cleaning cross-section. This thickness was found to be optimal for experiments at this accelerating voltage. Each of these steps was typically conducted at 1–5 min intervals, pausing to image the lamellae to reduce the influence of sample drift. The sample was imaged in the ion beam using a current of 1.5 pA between milling steps to realign as needed and to assess the quality and thickness of the finished lamella.

Setting up the Falcon 4 for diffraction experiments. The dose protector that prevents the operation of this detector while in diffraction mode was disabled by a service engineer before experiments. The Falcon 4 direct electron detector internally operated at 150 kV, 100 μA, 300 frames per second to monitor the detector and does not result in useful data. Owing to bandwidth limitations, the camera furthermore accumulates at least seven raw frames before transmitting their summed image to the controlling computer, which means that the user can obtain no more than ~35 images per second when collecting data in MRC format. For the data presented here, the frames were internally summed to correspond to either 1.0–0.5 or 0.5–0.25 wedge frames (120 or 119 raw frames).

The damage threshold for this system is described by a deterioration of the DQE rather than the number of events per unit time. Specifically, the DQE for each pixel will decrease by 10% after a total exposure of 1.5 × 10⁶ electrons. Ultimately, the smaller C₂ aperture of 50 μm was chosen, with a spot size of 11 and a beam diameter of 20 or 25 μm, corresponding to a total exposure range of 0.64–1.0 e⁻ /Å². Internally, counting mode operates by returning a single count per electron event that is then normalized by the post-counting gain reference. The resulting real pixel values in the MRC file correspond to unit gain and were multiplied by 32 and rounded to the nearest integer during conversion to SMV format. This is reasonably confirmed by the gain values, estimated between 30 and 36 during data processing. To estimate the number of electrons in each pixel in an individual MRC formatted image, we divided by 32 and rounded to the nearest integer. We simulated the pixel DQE in counting mode and mapped the corresponding values to a histogram of all the pixel values that we measured for the highest exposure dataset (Fig. 1d, Supplementary Code and Supplementary Fig. 1). Comparing the pixel values in the data to counts expected for a given MRC pixel that typical values fall within the linear region and that exposures are usually lower than those for single-particle movies (Supplementary Fig. 1). None of our measurements fell below a DQE of 0.6.

MicroED data collection. Grids containing milled protein crystals were rotated such that the TEM rotation axis was 90° from the FIB–SEM milling axis and then loaded into a Thermogenically coated electron beam TITAN T3030 with an in-column gas-injection system operating at an accelerating voltage of 300 kV. Low-magnification montages of each grid were collected at a magnification of 64× and used to locate the milled lamellae. Each lamella was brought to its eutectic position before data collection. MicroED data were collected by continuously rotating the stage at a rate of approximately 0.15° s⁻¹, or 36.3° s⁻¹, for 420 s, covering a total rotation range of 31.5° to 42°. This typically spanned the real space wedge corresponding to −31.5° to +42°. Data were collected using a 50-μm C₂ aperture, a spot size of 11 and a beam diameter of 20 or 25 μm. Under these conditions, the total exposure to each crystal per dataset was approximately 1.0 e⁻ /Å² or 0.64 e⁻ /Å², respectively. The exposure rate was confirmed by collecting an identically long exposure using the same beam size and settings in imaging mode in an empty grid square and collecting the movie in counting mode. This was repeated multiple times and averaged to measure the total exposure accurately. Diffraction data were isolated from a small area from the middle of each lamella of approximately 2 μm diameter at the specimen level using the selected area aperture of 100 μm to remove unwanted background noise. All data were collected using twofold binning and internally summed such that each image recorded either a 1.0- or 0.5-μs exposure spanning approximately 0.075–0.2° of rotation. In this manner, each image stack contained either 420 or 840 images, the last of which was discarded. A single sweep of continuous-rotation MicroED data was collected from each lysozyme lamella. For proteinase K, two sweeps were collected at a nominal camera diameter of 63° or 84°, and then a subsequent low-resolution dataset collected identically but at the longest possible nominal camera distance of 4,300 mm. The post-column magnification on this system is 1.81×. The low-resolution pass was conducted after the high-resolution pass covering a resolution range from approximately 60 to 5 Å.

MicroED data processing. Movies in MRC format were converted to SMV format using a parallelized version of the MicroED tools (https://cryoem.ucla.edu/downloads). Each dataset was indexed and integrated using XDS. All datasets were scaled using XSCALE and xscale_isoclu. Datasets were of either much poorer resolution or with scaling correlation below 90% were discarded. The uncertainty in unit cell parameters for the merged data was taken to be the standard deviation in the measured unit cells. For both crystals, the space group was verified using POINTLESS. For lysozyme, the data were merged without scaling using AIMLESS, the subsequent intensities were converted to amplitudes in CRUNCATE, normalized structure factors were calculated using ECALC, and a 5% fraction of the reflections was assigned to a free set using FREERFLAG software packages distributed in the CCP4 program suite.

Phasing. The lysozyme data could be phased using either Fragon or Phaser, followed by ACORN. An initial phasing solution was achieved using an electron density plot from the ACORN documentation (http://legacy.ccp4.ac.uk/html/acorn. html#example9). Here, a small fragment of idealized α-helix is used for molecular replacement, and then the best solution is subjected to density modification. In this manner, the number of atoms was systematically lowered in the idealized helix from the initial 50, and we were able to achieve a phasing solution with as few as 15 total atoms. Using fragments smaller than 20 atoms required placement using Phaser rather than the internal ACORN procedure. The resolution limits were also tested using the same 50-atom fragment, and the structure was solved using the loop reflection decay up to a resolution of 1.15 Å. The lysozyme model could also be solved in Fragon starting from a penta-alanine fragment, the smallest fragment allowed in the CCP412 interface.

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Modification, the protein was built automatically by Buccaneer20. For lysozyme, helical shapes and increasing the chain tracing rounds to 50 or 100. repeated 30 times. Among these trials, only the four placed 14-amino acid-long rounds of density modification are followed by standard chain tracing. This is SHELXE was run with or without the ‘-q’ option to first search for helix shapes density. From here, one, two and three copies of 14-amino acid-long helices were placed in space group 96. This was attempted again using ten-amino acid-long helices, for which only the search for four copies resulted in a convincing solution. SHELXE was run with or without the ‘-q’ option to first search for helix shapes during the chain tracing. The first solution from four helices in space group 96 used the SHELXE command line ‘sheelx 1.pda -q=0 -a=30’. Here, the default ten rounds of density modification are followed by standard chain tracing. This is repeated 30 times. Among these trials, only the four placed 14-amino acid-long helices gave an obvious solution after chain tracing. However, the solution in space group 96 with three helices placed was also able to give a similar solution upon adding the ‘-q’ option to trim away the low CC amino acids, ‘-q’ to search for helical shapes and increasing the chain tracing rounds to 50 or 100.

For both lysozyme and proteinase K after the last round of density modification, the protein was built automatically by Buccaneer22. For lysozyme, the entire protein was built into the map produced by ACORN23 except for two terminal residues that were not resolved upon inspection of the map in Coot24. For proteinase K, the traced backbone from SHELXE was used as a starting fragment for Buccaneer. In both cases, electron scattering factors were used for the maps.

The built structures were refined in REFMAC525 using electron scattering factors calculated by the Mott–Bethe formula. Initial refinements used isotropic atomic displacement (B) factors for individual atoms, and water molecules were added automatically. Refinement was always followed by manual curation of the model using Coot. For lysozyme, N2O ions were found in multiple locations that were not adequately modeled by single water molecules. For proteinase K, two J3C molecules with low occupancies were identified after the structure was entirely built and placed manually in Coot using the fragment code J3C. Hydrogen atoms were added to the lysozyme model in their riding positions for the final rounds of refinement. Once the model was completely built, the models were refined again using anisotropic atomic displacement parameters for all but the hydrogen atoms.

Simulating the DQE for a single pixel during 250 frames. The simulated DQE is calculated as the ratio of the number of counted electrons, Ncount, and the number of incoming electrons, Ninv. It is assumed that the detector does not overcount, that is, Ncount ≤ Ninv and DQE ≤ 1. However, if two or more electrons arrive during the same frame, undercounting occurs because the pixel records at most one electron per frame. Over a 250-frame interval, Ncount will be equal to the number of frames that recorded at least one electron. By choosing the frame randomly from a uniform distribution in the range (1, 250) for each of the Ninv incoming reflections, the DQE can be simulated. To establish the DQE curve, the 250-frame exposure was simulated 1,000 times for each value of Ninv. This code is available in the Supplementary Code.

Comparisons of MicroED and X-ray structures. The observed amplitudes for triclinic lysozyme determined here and by X-ray diffraction to similar resolutions were compared (Supplementary Fig. 4). Observed structure factor amplitudes were compared against those from PDB 4LZT26. The calculated structure factor amplitudes were calculated using the proper scattering factor libraries using SFALL27. Intensities from the deposited X-ray structures were converted to amplitudes using CTRUNCATE28. Cross-correlation scatterplots with best fit lines and the correlation coefficients between datasets were calculated using Microsoft Excel.

Statistics and reproducibility. Twenty crystals of lysozyme and five proteinase K crystals were identified and milled. Of these, 18 lysozyme crystals and four proteinase crystals survived milling and transfer between the FIB–SEM and TEM. All lamellae diffracted, and data were collected. Of those collected, two lysozyme datasets and two proteinase datasets were discarded during the merging stage for deteriorating the quality of the merge. For simulation of the camera DQE, simulations were repeated 256 times and averaged. The standard deviation is given as a band in the plot of Fig. 1d.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41592-022-01485-4.

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Competing interests

The authors declare no competing interests.

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Author contributions

T.G. and M.W.M. designed the research. M.W.M. and T.B.C. prepared samples. M.W.M. and M.T.B.C. collected data. M.W.M., M.T.B.C. and J.H. analyzed data. M.W.M., M.T.B.C., J.H. and T.G. wrote the manuscript and prepared figures. T.G. managed the project.

Competing interests

The authors declare no competing interests.

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  
  *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*

- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  
  *Give P values as exact values whenever suitable.*

- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: FIB/SEM data were collected using the user interface software, microscope control v13.4.2 of a ThermoFisher Aquilos system. MicroED data were collected manually via the camera user interface software, VELOX v3.0.0 on a ThermoFisher Krios 3Gi transmission electron microscope.

Data analysis: XDS version Feb 5 2021 BUILT=20210323
PHASER 2.8.3
ACORN v2
CCP4 7.1.016
REFMAC v5.8.0049
MicroED tools v0.0.3 build 20201231
SHELXE v2019/1
BUCCANEER from CCP4 build 7.1.016

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  20 lysozyme (n=20) and 5 proteinase K (n=5) crystals were selected for this study. Samples were selected based on their random availability upon the EM grids. Successful ab initio phasing and structure solution dictated that no further samples were required for this study. No sample size calculations were performed prior to data collection.

Data exclusions  Crystal datasets were discarded if they scaled with a correlation coefficient below 95% with other datasets. Two lysozyme datasets and two proteinase K datasets were discarded.

Replication  Small variations may arise in crystal growth and crystals deteriorate during data collection due to radiation damage. Therefor, each crystal was measured only once. Repetition of n=20 and n=5 for the two samples served as the only form of replication conducted.

Randomization  Test reflections equally 5% of the total reflections in each scaled dataset were randomly generated for validation. Calculation of random half datasets was conducted using standard crystallographic software listed herein without user intervention. Randomization of crystal samples is not relevant to structure determination studies.

Blinding  Investigators were not blinded to group allocation as no groups were allocated in this study.

Reporting for specific materials, systems and methods

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Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology and archaeology |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |
| ☒   | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChiP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |