Effects of Freezing on Protein Solutions

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Introduction

When one tries to solve the structure of a protein by using x-ray crystallography, it is customary to flash freeze the crystal to liquid nitrogen temperature before exposing it to the powerful x-ray synchrotron beam. This procedure has been shown to reduce the damage to the crystal caused by radiation exposure. We used the flash freezing method to protect small crystals from radiation damage during an attempt to measure the coherent x-ray diffraction (CXD) [1] pattern of crystals of the protein ferritin obtained from equine spleen. During this experiment, a protein aggregate state was found to appear upon freezing of the solution. It was decided that this state warranted further investigation. We report on our findings on the aggregation of ferritin upon freezing here.

Methods and Materials

Equine spleen ferritin was purchased from the Sigma-Aldrich Company. It consists of a spherical protein shell and an iron core containing up to 4500 iron atoms; thus, it scatters strongly at small angles. The samples were solutions of 100 mg/mL of ferritin in 150 mM NaCl in aqueous solution buffer at pH 7.

The samples were placed in quartz capillaries that were glued onto a peltier cooling stage by using thermal paste. The peltier cooler was mounted on a water-cooled copper block used to cool the hot side of the peltier. This setup allowed temperature to be varied between -40°C and 20°C. This is far above liquid nitrogen temperature, but it was found that this is the interesting temperature range and that the aggregated state seen at 80K forms at a much higher temperature close to the freezing point.

Once the samples were mounted on the cold stage, they were placed in the monochromatic x-ray beam at UNI-CAT beamline station 34-ID-C at the APS. The small angle x-ray scattering (SAXS) patterns were measured by using a charge-coupled device (CCD) detector located 1090 mm from the sample. The collected images were integrated by using the FIT2D program [2].

Results

At temperatures above freezing, the SAXS patterns showed a monotonic decrease consistent with polydisperse spheres. This is expected for iron-containing ferritin, because the shape and size of the iron core varies from protein molecule to molecule, unlike the spherical shells, which are identical. As the temperature is lowered below the point where the sample appears to freeze when it becomes opaque, the SAXS pattern changes, and a large broad peak appears. The position of this peak in reciprocal space changes as the temperature is decreased further. The peak moves to larger q, as can be seen in Fig 1. The behavior is reversible at least a few times before radiation damage causes the whole signal to disappear.

We fitted all these integrated patterns and obtained a position and width for each peak. From the position, we can get a length scale, which, for lack of a better word, we call a lattice parameter. The peaks seen are broader than Bragg peaks from ferritin crystals but much narrower than amorphous peaks. They are then indicative of some form of aggregation of the protein that is between amorphous and crystalline. The value of the length scale extracted from each SAXS curve is plotted versus temperature in Fig. 2. The aggregated state of the protein displays dramatic thermal expansion over a small temperature range. The spacing measured increases by more than 25% over a range of 30°C. The process was found to be reproducible for a few temperature cycles, which indicates that the proteins are not destroyed upon freezing.

The structure of the observed aggregated state upon freezing is not well understood at this point in time. The

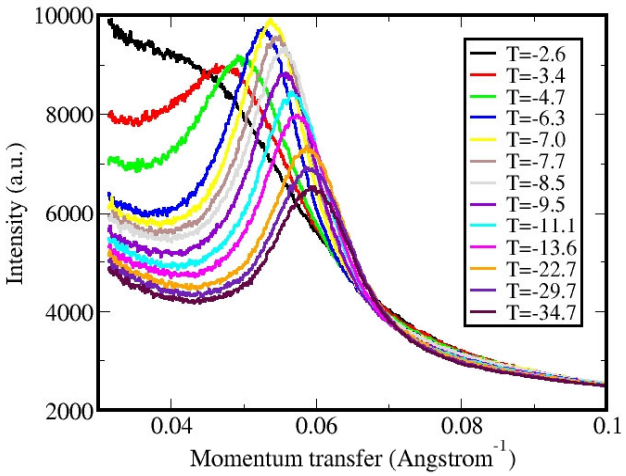

FIG. 1. SAXS integrated curves of ferritin solutions at different temperatures. A large peak in the intensity moves to a smaller q at a higher temperature.

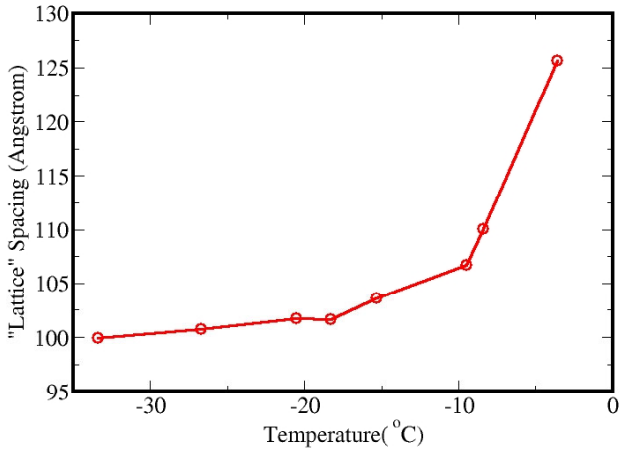

FIG. 2. Thermal expansion of the ferritin aggregated state. The "lattice" spacing corresponding to the peak position of the SAXS pattern is seen to vary by more than 25% over a range of only 30°C.

width of the peak in the intensity indicates that it is neither amorphous nor crystalline. We use these widths measured to obtain an average aggregate size of roughly 250 Å. This value was found to be rather independent of temperature, as shown in Fig. 3, except at the highest temperature measured (just below the melting point), where the sample reverts to a homogeneous solution and no peak is seen in the SAXS intensity.

Discussion

The fact that freezing solutions of ferritin caused some aggregation was a surprising result to us. Furthermore,

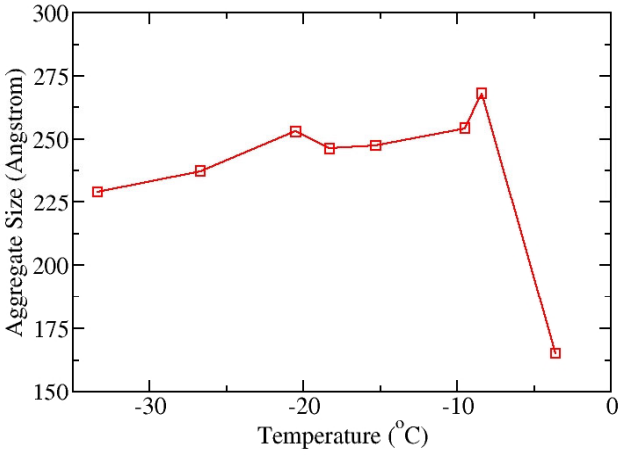

FIG. 3. Variation of the size of the aggregates with temperature.

this aggregated state does not seem to correspond to the standard fcc crystal structure of ferritin crystals. The lattice spacing of the first Bragg reflection of these fcc crystals is 106 Å, which is larger than some of the spacings measured and shown in Fig. 3. We would like to think that this aggregated state is a precursor of crystallization, but this may not be appropriate, since one state does not apparently transform into the other. Since no cryoprotectant was used in the solutions, it is possible that upon the formation of ice in the sample, ice crystal networks are formed, leaving voids. The ferritin would then be constrained within these voids and might phase-separate from the solvent. It might then aggregate as a result of concentration in the small volume in which it is confined.

As the temperature is lowered, these voids can become smaller as more ice forms, thus compressing the aggregates further. This could explain the large packing change as the melting point is approached. However, the thermal expansion at temperatures below the point where the sample appears completely frozen cannot be explained by the thermal expansion of ice. One possible explanation is that as ice crystals shrink upon cooling, they exert pressure on the aggregates inside the voids. It is well-known that protein crystals are very soft when compared to inorganic crystals. Thus, only a small change in pressure exerted on the protein aggregates could lead to large changes in the spacing. A small thermal expansion of the ice crystals can therefore lead to a much larger apparent thermal expansion of the protein aggregates.

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