NUCLEATION OF INSULIN CRYSTALS IN A WIDE CONTINUOUS SUPERSATURATION GRADIENT

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Nucleation is a key step in the crystallization process. Being its first stage, it predetermines to a great extent many features of the subsequent crystal growth. The nucleation rate, for instance, determines the number of crystals that may grow. Unfortunately, due to the inherent difficulties in monitoring the nuclei themselves, our present knowledge about the nucleation stage in protein crystallization is insufficient.

Separating the nucleation and growth stages allows direct determination of the nucleation rates. Strict separation in time of these stages lies at the root of the classical approach for determining nucleation rates at a given supersaturation. The principle is very simple (Fig. 1a). During the first stage, of higher supersaturation, the crystals are (predominantly) nucleated. By keeping this period relatively short, the crystals do not have enough time to grow. If it is too long Ostwald ripening may occur. After the (expected) onset of nucleation, the supersaturation is rapidly lowered to below the threshold which is necessary for nucleation, into the so-called metastable zone, so that no further nuclei may appear during that second (growth) stage. Only the existing nuclei grow into crystals during the second stage, which is made long enough for crystals to become visible. The crystals are then counted under an optical microscope. The stationary nucleation rate is obtained simply by plotting the number \( n \) of crystals grown to visible sizes vs the nucleation time, \( t \). The technique is usually called double pulse technique. It enables the measurement of rates of nucleation experimentally, without ever actually seeing the nuclei themselves.

In the present work we use the modification of the double pulse technique, which principle is depicted schematically in Fig. 1b. Instead of moving from one starting (nucleation) value for the supersaturation to an end (growth) value, we have produced a continuum of starting (nucleation) supersaturation levels, using a temperature gradient. Thus, several measurements of nucleation rates were performed at once, for different values of the supersaturation. The supersaturation gradient is applied along a (cylinder of) protein solution contained in a glass capillary tube (the “nucleation” area in Fig. 1b). At every point of the capillary tube the supersaturation is kept constant throughout the entire nucleation time period, \( t \), and in all experiments of a given series. Temperatures were kept fixed, at 5°C and 23°C, at the two ends of the capillary.

One of advantages of this apparatus is when only limited protein amount is available, in order to limit protein consumption, we use supersaturation gradient. To demonstrate the
applicability of this technique we performed experiment with insulin since temperature
dependence of its solubility is available. Solubility data for this protein were taken from prof.
Vekilov’s group.

A glass capillary tube was graduated in 5 mm segments (18 segments in all). It was then
filled with insulin crystallization solution and placed horizontally in the gradient for various time
intervals $t$. At the end of the pre-set nucleation time, the capillary was exposed to a constant
temperature of $23 \pm 0.5^\circ C$. The supersaturation was thus substantially lowered, and kept constant
both in time and along the capillary during the growth stage (Fig. 1b). Growth for approximately
two days yielded visible crystals (Fig.2). The number of crystals in each segment was then
counted and plotted as a function both of $t$ and of the (initial, i.e. nucleation) supersaturation. This
procedure was performed repeatedly for each $t$ in order to yield reliable statistics, since the
stochastic character of the nucleation process means a high inherent data scatter.

Still another advantage is that the nucleation time lag has been measured directly and
readily. This is done by simple microscopic inspection: The capillary was scanned and the
supersaturation below which nucleation did not take place during this particular nucleation time
was recorded. Thus, the time lag has been determined without using $n$-$t$ curve, independently in
every measurement.

![Fig. 2 Rhombohedral insulin crystals.](image)

![Fig. 3 Plot of $t$ vs $\mu/k_B T$. The dependence is linear.](image)

The rationale underlying our procedure are as follows: Provided that sufficiently long
time is awaited, crystal nucleation only took place in the parts of the capillary where the
supersaturation was greater than that corresponding to the upper limit of the metastable zone. Note
that we do know this limit. It starts at about $s=1.29$, and was established in preliminary
experiments, simply by presence or absence of insulin crystals.

But we observed that the shorter the nucleation time we chose the longer is the part of the
glass capillary without crystals, including segments where the supersaturation is above that of the
upper limit of the metastable zone. The observation means that the time chosen is insufficient for
nucleus formation. This is proven by the fact that for longer times we do observe crystals, created
also under these lower supersaturations. In practice, we recorded the supersaturation segment
where the last crystal was "born" during the nucleation time $t$ chosen by us. Consequently, we
observed that the time lag $t$ is supersaturation-dependent. The higher the supersaturation, the
shorter the time that appears to be sufficient for the creation of at least one cluster of critical size
(i.e. a critical nucleus), Fig. 3.

In agreement with general expectations, the number $n$ of nucleated crystals increases
exponentially with supersaturation, along the capillary. We started (successfully) to plot $n$ vs. $t$. A
linear dependence is obtained for the two highest supersaturations applied in our study.

This technique can be used with any protein, provided that the temperature dependence of
its solubility at given conditions (protein and precipitant concentration, pH, etc.) is known.