Screening crystallisation conditions using fluorescence correlation spectroscopy

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We investigate the potential of fluorescence correlation spectroscopy (FCS) in screening for crystallisation conditions. Solutions that nucleate protein crystals must have different interactions than solutions that do not give rise to crystals. Due to these different interactions the average mean squared displacement of the individual proteins changes. By monitoring protein self-diffusion, we can distinguish crystallising from non-crystallising solutions. The method introduced can be applied at extremely low concentrations in femtoliter volumes as an early diagnostic for molecular association. Based on our preliminary findings FCS has the potential to become a routine screening method for crystallography.

Keywords: diffusion; lysozyme; fluorescence correlation spectroscopy; crystallisation conditions

1. Introduction

High throughput screens to find the optimal conditions for protein crystallisation are being developed in a number of laboratories to keep up with the pace of production of soluble protein by different structural genomic projects. There is a clear trend towards miniaturised robotic systems with automated crystal detection (Stevens, 2000; Luft *et al.,* 2001; Mueller *et al.,* 2001; Kuil *et al.*, 2002). This approach will allow for a far more systematic study of the process of protein crystallisation in view of the larger range of experimental conditions that can be explored. The extensive automation of the liquid handling increases the reliability and reproducibility of the obtained results (Stewart & Baldock, 1999).

We know that a protein molecule that is incorporated in a protein crystal shows a largely reduced motion when compared to a protein moving freely in solution. We were therefore curious to find out whether the mean square displacement of proteins in solution leading to crystallisation is different from that in non-crystallising solutions. The average mean square displacement can be related to the self-diffusion of a molecule. We use fluorescence correlation spectroscopy (FCS) to measure the self-diffusion of fluorescently labelled proteins. The influence of the increasing concentration of protein could be studied by looking at a very low concentration of labelled protein. In all cases it was observed that the motion of the labelled protein is reduced by the presence of increasing amounts of unlabelled protein.

The study of dynamics in concentrated solutions is of great interest to protein crystallographers and is also needed to understand many intercellular processes (Minton, 2001).

In FCS the fluorescence fluctuations in the focal volume of a microscope objective are recorded. This volume is roughly 1.5 fl (10^{-15} liter) in our system. The fluorophore concentration is chosen such, that only one or at most a few molecules are observed at a time. The fluorescence signal depends on the number of fluorescent molecules in the focal volume, their diffusion properties, and their photophysical properties. The fluctuations in the fluorescence signal are dominated by the Brownian motion of the molecules, and can be used to determine the average residence time of a diffusing particle in the focal volume. The self-diffusion coefficient can be derived from this residence time. The use of fluorescence correlation spectroscopy to monitor diffusion and association was already proposed in the seventies (Magde et al., 1972; Ehrenberg & Rigler, 1974) and has gained popularity the last years (Schwille et al., 1997). To exploit the full potential of the technique, we selected a fluorescent probe that has a high quantum yield for fluorescence and a strong absorbance band in the red part of the spectrum to avoid interference with chromophores naturally occurring in proteins.

To avoid any confusion, we note that the diffusion coefficient that is determined in most dynamic light scattering experiments is the cooperative diffusion coefficient, which depends on the thermodynamics and the hydrodynamics in the solution (Kops-Werkhoven et al., 1982). With FCS we measure the self-diffusion coefficient. The self-diffusion coefficient can be calculated from observations of the trajectory that a single molecule follows in a solution kept at a constant temperature. For very dilute solution the self-diffusion coefficient is virtually identical to the cooperative diffusion coefficient.

To correctly describe the diffusion in terms of solute flows in multi-component solutions (cooperative diffusion) one should use the generalised diffusion equation to take into account the coupled flow of the different components (Gosting, 1956, Tanford, 1961, Fu 2002). Data pertinent to multi-component diffusion of proteins is reported by Albright and co-workers and indicates that inclusion of the cross terms describing the coupling of the transport of lysozyme and the electrolyte is essential (Albright et al., 1999; Annunziata et al. 2000). In contrast, FCS allows us to study the dynamics of the protein component only.

Fluorescence correlation spectroscopy is generally performed at extremely low density of fluorophores that the self-diffusion of the label is measured. In general the self-diffusion coefficient is reduced when the concentration is increased; the cooperative diffusion increases with increasing concentration when repulsive interactions are dominant (Berne & Pecora, 1976). For solutions that are close to crystallisation, the cooperative diffusion is a decreasing function of the protein concentration (Rosenberger et al., 1996; Beretta et al., 2000).

2. Material and methods

2.1. Protein labelling

All proteins were covalently labelled through surface accessible amino groups with Cy5 mono-functional succinimidyl ester (Amersham Biosciences, Uppsala, Sweden), as shown in figure 1B. Details on the labelling chemistry used are published in e.g. Haugland (1996). The Cy5 fluorophore, a cyanine dye, is covalently attached to the protein using the conjugation protocol suggested by the manufacturer. Briefly: Cy5 succinimidyl ester was dissolved to a final concentration of 6 mM in DMSO. 0.5 mg protein was dissolved at 5 mg/ml in 0.1 M Na_2CO_3 buffer pH 8.3. Dye and protein solutions were mixed with a volume ratio of 1:19. After ~ 1h incubation at room temperature free dye was removed with a Centri-Spin 10 size-exclusion chromatography column (Princeton Separations, Adelphia, USA). Solvent conditions (e.g. pH) can be changed in this step. Labelling ratios were determined spectrophotometrically to be between 0.3 (lysozyme) and 1.6 (apo-ferritin) dye molecules conjugated per protein molecule or protein oligomer (e.g. on average 1.6 dye molecules per apoferrin 24-mer). After the purification step, no free dye was detected in the lysozyme and ovalbumin preparation (data not shown). When appropriate, a

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two component fit was applied in evaluating the FCS data to take free dye into account. The Cy5-myoglobin conjugate lost fluorescence after 2-3 days, most likely due to quenching by the protein or the haem group. All other conjugates were stable for several weeks when stored at 4 ºC. Two different commercially available lysozyme preparations were used (Sigma (Zwijndrecht, The Netherlands) and Roche (Basel, Switzerland)). All other proteins were obtained from Sigma, and were used without further purification. The chosen proteins have already been crystallised and studied extensively, including their oligomerisation in solution (see e.g. Steinrauf 1959, Ries Kautt & Ducruix 1989, Stein et. al. 1991, Folta-Stogniew & Williams, 1999).

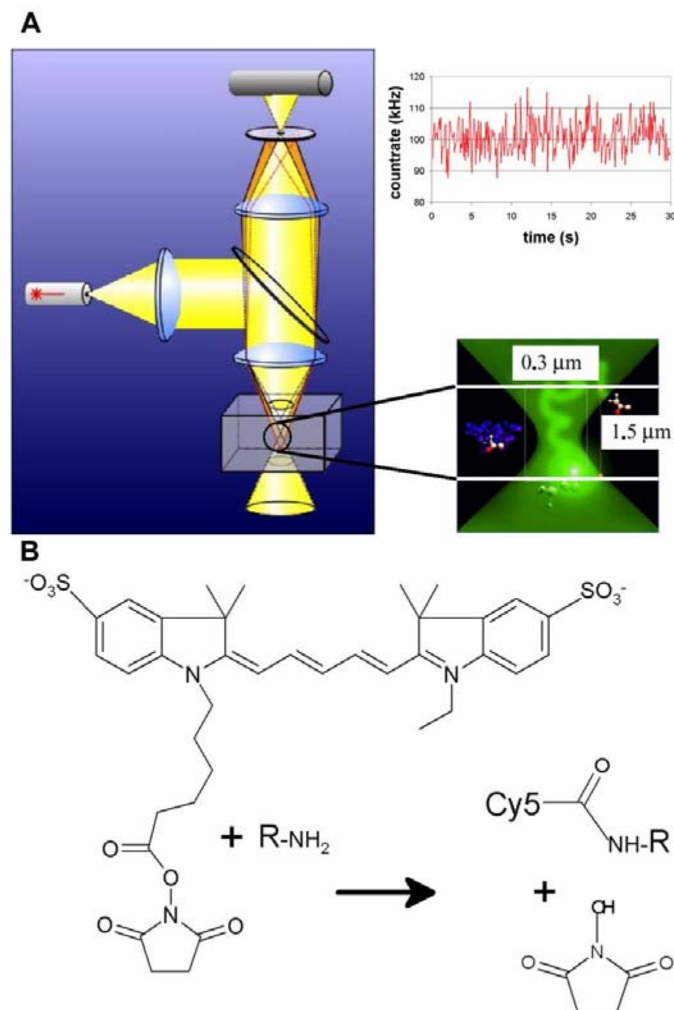

Figure 1

A: A FCS-setup is similar to a laser scanning microscope. Instead of scanning and measuring the fluorescence on different positions, the FCS system is monitoring the fluorescence intensity fluctuations, due to Brownian motion of labelled molecules in and out of an immobile confocal spot. The inset shows an example of recorded raw data. **B:** Labelling proteins with a fluorescent dye: the Cy5 succinimidyl ester derivate reacts with free amino groups on the protein.

2.2. FCS-measurements

The setup is schematically shown in figure 1A. A HeNe-laser (633nm) is coupled *via* a dichroic beam splitter into the beam path of a microscope objective and focussed to the diffraction limit. Fluorophores in the beam are excited and emit red-shifted light in all directions. Part of this light is collected by the objective while scattered excitation light is removed by a filter. A pinhole excludes the out-of-focus light. Photons are detected by an avalanche photodiode and the autocorrelation function is derived from the intensity fluctuations by software correlation.

All FCS-measurements were carried out using a Confocor2 System (Carl Zeiss, Jena, Germany). After pinhole adjustment, residence times for labelled protein at low concentration (1-10 nM) were established to compensate for day to day drift in the system. For the measurements with changing protein concentration all values were normalised using these residence times. The free dye residence time was measured during each run as a control.

Labelled protein was added to solutions of unlabelled protein at various concentrations to a final fluorophore concentration of ~ 5nM equivalent to 1- 1.5 fluorophores in the confocal volume on average. Typically, a volume of 40 µl, sufficient for quantitative manual pipetting, is used for measuring. Much smaller volumes are possible with appropriate liquid handling. After illuminating for 10 s (to remove possible immobile fluorophores by irreversible bleaching) five independent measurements of five seconds each were recorded.

2.3. Data evaluation

The recorded auto correlation functions, *G(t)*, were fitted using the software supplied with the system. Data are fitted to:

$$G(t)=\frac{1}{N}\left(1+\frac{T}{1-T}e^{-\frac{t}{\tau_T}}\right)\sum_{i=1}^{M}\frac{f_i}{1+\frac{t}{\tau_{D_i}}}\frac{1}{\sqrt{1+\frac{t}{S^2\tau_{D_i}}}}+1 \qquad (1)$$

(N = number of particles, T fraction of fluorophores in triplet state, τ_T triplet lifetime, M number of fluorescent components, f_i fraction in this component, τ_{Di} residence time, S structural parameter describing the confocal volume) (Kettling *et al.,* 1998). This model includes a term for triplet states of the fluorophores, which also accounts for the impact of the cis/trans isomerisation of Cy5 on *G(t)*. Fitting of a meassured Cy5 correlation curve with the full model from Widengren & Schwille (2000) gave identical results for the residence times. The structural parameter and residence time of the free dye were established in a separate measurement and kept fixed during analysis of the data obtained. To establish if more than one component was present, all data were evaluated with a two-component model (M=2). If the two residence times were identical or if one of the fractions was less than 3%, a single-component model (M=1) was imposed.

We focussed our attention to the average residence time or "diffusion time" of a fluorophore in the confocal volume.

The residence time is related to the diffusion constant D by

$$\tau_D=\frac{\omega_1^2}{4D} \qquad (2)$$ where ω_1 is the laser focus radius.

The value for ω_1 can be calibrated with a compound with known D. We found the impact of refractive index changes on the residence time, due to different salt and protein concentration (*via* a changed confocal volume), to be negligible (< 3%).

3. Results

3.1. Very dilute labelled protein solutions

We labelled a number of proteins with the Cy5 fluorophore. The ratio of dye to protein was between 0.3 and 1.6 dyes per protein. This low labelling ratio ensures that on average most proteins will

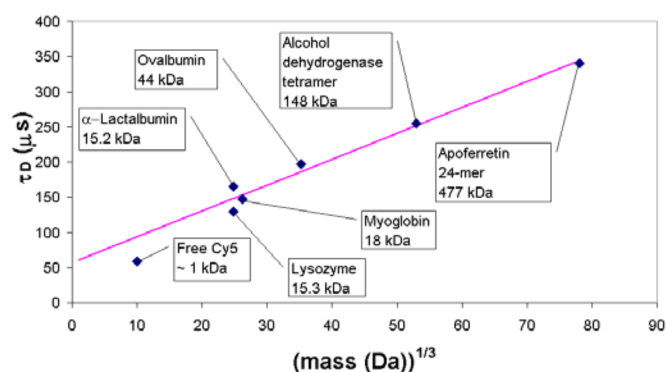

Figure 2

Residence times of Cy5 labelled proteins. Even while very different globular proteins were used, the residence times scale with (molecular mass) $^{1/3}$. Protein concentrations were between 5 and 50 nM. At this low concentration self- and cooperative diffusion are indistinguishable and interactions between proteins can safely be ignored.

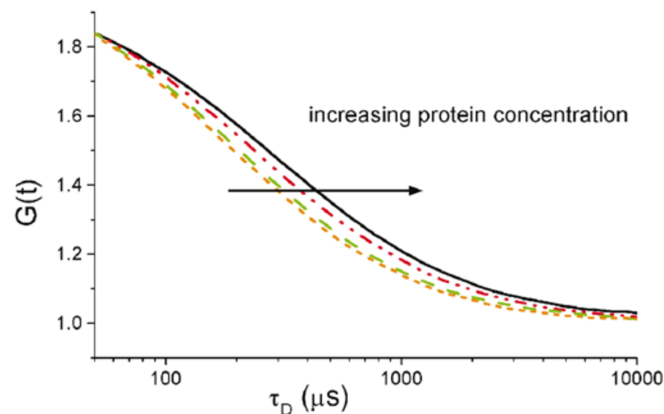

Figure 3

Cy5-labelled lysozyme (~5 nM) in an excess of unlabelled lysozyme in 50 mM NaOAc pH 4.5, 1M NaCl. This is part of the raw data to the "1M salt" curve in figure 4A. Fitted residence times are: 276 µs for 1.6 mM (—), 225 µs for 0.8 mM (- ··), 180 µs for 90 µM (––) and 162 µs for 0.45 µM (--).The highest two concentrations were crystallising. The curves were normalised at 15 µs to allow a better visual comparison.

have only one label, avoiding complications due to interactions of the fluorophores.

Figure 2 shows the observed relationship between diffusion and molar mass of the proteins. In this experiment the protein diffusion in very dilute solutions is studied, where self- and cooperative diffusion is indistinguishable. The concentration of the labeled protein was always adjusted to ensure an average concentration of one label in the focal volume. We observed the residence time to scale approximately with the cubic root of the protein mass as predicted for homogeneous spherical particles (*(2)* and Tanford 1961). The oligomer of apo-ferritin has an observed diffusion that is in agreement with the presence of a 24-mer. This oligomer is stable for at least a week at nanomolar concentration (data not shown.). For alcohol dehydrogenase we assume that the molecule is a tetramer. This is probably not the equilibrium configuration at nanomolar concentrations since the tetramer slowly dissociates into smaller units after a few hours at the FCS concentration. We could determine the self-diffusion coefficient very efficiently using FCS in samples with concentrations of below 10 nM labelled protein. In all we labelled less than one milligram of protein, which would allow us to perform 10^5 FCS experiments at the concentrations and volumes used in this study.

3.2. Increasing the protein concentration

When the protein concentration is increased the self-diffusion coefficient is expected to decrease due to the reduction of the free volume available for diffusion. In our experiments the situation is somewhat more complicated: we increase the protein concentration by adding unlabelled protein, which might display a slightly different physical chemistry compared to the labelled protein. We can only draw definite conclusions about the labelled protein since this is the protein that is observed in the experiment. A typical set of fluorescence autocorrelation functions measured for increasing protein concentrations is shown in figure 3.

In figure 4A we show the concentration dependence of the residence time of lysozyme for three different concentrations of added NaCl. We varied the protein concentration over more than four decades, a concentration range that has never been covered before, using FCS. Below volume fractions of 0.01% no significant change in the observed residence times is observed. This indicates that there is no specific interaction between the labelled protein alone and the unlabelled protein at these concentrations. This observation is crucial, because it indicates that the behaviour of the labelled protein is a valid reporter for the solution properties (Vink, 1985). Note that at the lowest concentration included in figure 4a the ratio between labelled and unlabelled protein is already 1 labelled part in 100 unlabelled parts.

We have compared the observed increase of the residence time with a theoretical model that describes the self-diffusion of hard spheres (Tokuyama & Oppenheim, 1994). We observed that the tracer protein appears to be slowed down much more rapidly upon increasing the protein concentration than predicted theoretically. The hard sphere model has no unknown parameters, the volume fraction can be derived from radius of the protein which can be determined in an independent experiment. In our interpretation we ignore any differences between the labelled and unlabelled proteins.

Alternatively we could consider the labelled protein dissolved in a solvent with increasing viscosity built by the presence of unlabelled protein. In this case we could use the theoretical prediction for the viscosity of a hard sphere fluid (Batchelor, 1977). This model also does not describe our data accurately (see figure 5). At 1.0 M NaCl the two most concentrated protein solutions showed crystallisation within 24h. Both the hard sphere model and the viscosity model do not take into account specific interactions between proteins apart from the volume occupied by the proteins and their hydrodynamics in the solvent.

We also tested two other mother liquids that are known not to promote crystallisation. Lysozyme in a buffer with ammonium sulphate as the added salt at a higher pH (10 mM Tris-SO_4, pH 7; see figure 4B), and ovalbumin in 10 mM HEPES, pH 8.1 with 100 mM sodium nitrate (see figure 5). The lysozyme charge is lower at pH 7 and the protein is known not to crystallise in solutions with sulphate as the anion without further treatment (Riès-Kautt *et al.*, 1994).

Again a very strong dependence of the self-diffusion on the total protein concentration is observed. The concentration dependence of the ovalbumin self-diffusion was much less pronounced than for lysozyme (figure 5) and the results are in good agreement with self-diffusion data of some other globular proteins in solutions with moderate amounts of added salt (Le Bon *et al.*, 1999). As a further

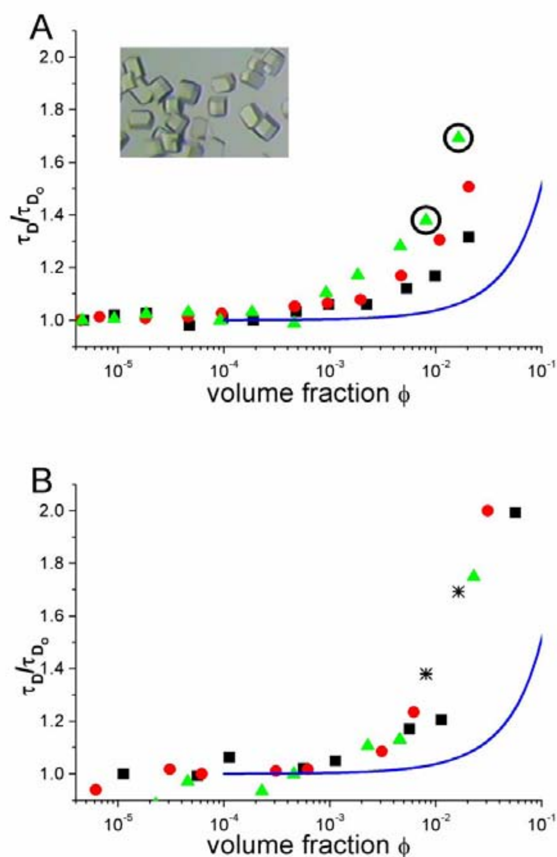

Figure 4

A: Effect of increasing protein concentration on the self-diffusion of labelled lysozyme in different salt concentrations(no NaCl (squares), 0.31M (circles) and 1M (triangles)). Buffer: 50mM NaOAc, pH 4.5 and NaCl. The two highest protein concentrations (emphasised) were crystallising (in less than 24 h). Inset: picture of the crystals formed during the measurements. To illustrate the scale: the lower crystallising protein solution had a concentration of 0,81 mM, which equals 11,5 g/l or 0,81% Volume fraction. **B:** Control experiment: 10 mM Tris-SO_4, pH 7 and $(NH_4)_2SO_4$ was used (no $(NH_4)_2SO_4$ (squares), 0.2M (circles) and 1M (triangles)). The slopes are similar, to figure a), but no slope is steeper than the crystallising condition in A (*). In A and B the hard sphere model is plotted as a comparison.

control lysozyme was also measured in the HEPES buffer with 100 mM sodium nitrate. Again a similar, strong, concentration dependence as in the other lysozyme experiments was found indicating that the rapid slowing down as a function of concentration is not buffer dependent (figure 5). No crystals were formed in all three systems.

The simplest explanation of the stronger concentration dependence of the self-diffusion in lysozyme solutions is the occurrence of a concentration dependent aggregation process. It is tempting to apply a model to describe such aggregation process. This is not possible with the standard data analysis programs used in FCS and in addition specific assumptions on size and shape of the aggregates have to be made for such analysis.

The observed increase in residence time is at least partly due to obstruction by other protein molecules. The effect of association on this obstruction has never been studied theoretically. We note that the hard sphere model alone cannot explain our observations for ovalbumin, where we do not expect aggregation. The hard sphere model has been applied successfully to describe the self-diffusion of myoglobin up to very high volume fractions (Nesmelova & Fedotov, 1998) but fails to describe aggregating systems.

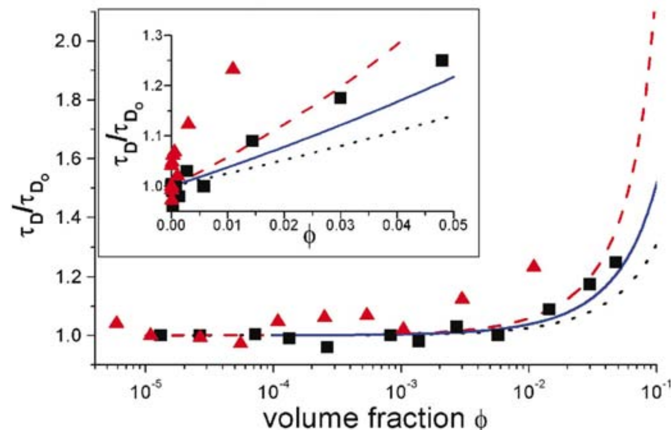

Figure 5

Data from various concentrations of ovalbumin (squares) and lysozyme (triangles) in 10 mM HEPES, pH 8.05 with 100 mM $NaNO_3$. While the ovalbumin data is corresponding nicely to other data from literature (dashed line), neither the hard sphere model (solid line), nor the viscosity model (dotted line; $\tau_D/\tau_{D0}=1+2.5\phi+6.2\phi^2$) is describing the data well at higher concentration. The inset shows the same data magnified and on a linear scale.

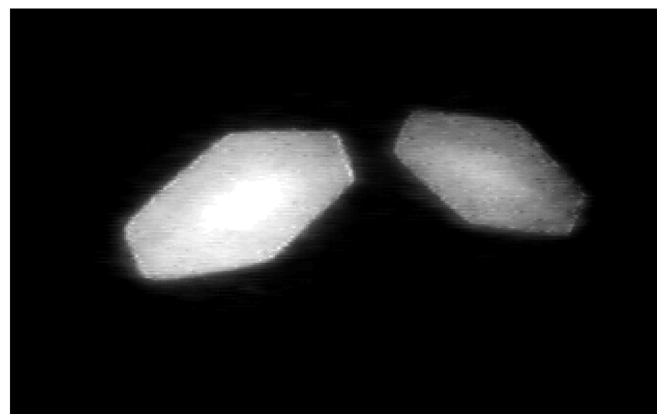

Figure 6

Fluorescence microscopy picture showing that Cy5-labelled lysozyme is homogeneously incorporated into tetragonal crystals. In transmitted polarised light microscopy the crystals were behaving as unlabeled protein crystals grown under identical conditions. With a volume of about 200 μm^3 the crystals were too small for X-ray diffraction experiments.

3.3. Crystallisation

It is well known that contaminants can compromise the crystallisation of proteins (see for instance Rosenberger, 1996). In our FCS experiments is it very unlikely that the labelled protein can influence the crystallisation since the ratio of labelled to unlabelled protein is at least $1:10^5$ in the solutions that showed crystallisation. This contamination is much lower than contaminations that are

normally detected in any protein crystallisation experiment. It could be argued that the labelled protein does not incorporate in the formed crystals or associates differently with unlabelled protein. To rule out this possibility we co-crystallised lysozyme with Cy5 labelled lysozyme, where approximately one out of hundred molecules was labelled. The labelled protein was incorporated homogeneously into the crystals (figure 6). In a control experiment using only unlabeled protein, crystals of similar size and shape were obtained. The average distance between the labelled molecules should be large enough to rule out self-quenching of the fluorophores.

4. Discussion

Results reported in this paper show the concentration dependence of protein self-diffusion in concentrated solutions. It has to be pointed out, that there is no trivial relation between self- and cooperative diffusion known for concentrated solutions. Therefore theories on cooperative diffusion can not be directly applied to self-diffusion data. FCS and dynamic light scattering determine a diffusion coefficient. The derived experimental quantities can not be related to protein crystallisation in a simple manner and further research is needed. Using both techniques on the same experimental system may increase our understanding of protein crystallisation.

Our results are in qualitative agreement with observations made with pulsed field gradient NMR on crystallising solutions of lysozyme. However, we observed a much stronger reduction of the self-diffusion than Price *et al.* (1999, 2001). It should be noted that there are important differences between the conditions used in NMR as compared with FCS. Apart from the presence of a strong homogeneous magnetic field known to influence the crystal growth (Sato *et al.,* 2000, Lin *et al.,* 2000) often a percentage of the water is substituted by deuterium oxide for technical reasons. It cannot be ruled out that the introduction of a modified solvent (e.g. a H_2O/D_2O mixture) and/or the strong magnetic field influences the crystallisation and thus the observed diffusion. On the other hand the labelling of lysozyme with the Cy5 label increases the molar mass of the conjugated protein and will surely alter some of the protein-protein interactions. It is, in general, not known which protein-protein interactions are dominantly affecting the protein crystallisation process. The impact of the added dye should perhaps be compared to the effect of mutagenesis: some mutants crystallise, some not. In the case of mutagenesis all molecules in the ensemble are identically modified, in our case only a very small fraction of molecules is modified, the label is not always bound to the same residue of the protein, and we expect the impact to be relatively low.

The most likely explanation for the discrepancy between our results and the pulsed field gradient NMR results is that the presence of larger aggregates is underestimated in the NMR experiment (Price *et al.*, 2001). The increase in viscosity in crystallising solutions in high magnetic fields as claimed by Zhong & Wakayama (2001) may even enlarge the discrepancy between the results.

Using FCS we have shown, that we can easily detect associates of 470 kD (see figure 2) and observed in a time series an increase in the residence time for the crystallising solutions (data not shown) indicating the growth of (crystalline or non-crystalline) associates present in the solution. As the FCS signal in crystallising solutions corresponds to fairly small associates shows that the method has potential as a detection of crystallites in solution that cannot be detected using conventional light microscopy.

Fluorescence microscopy images suggest that the Cy5 labelled protein incorporate homogeneously into the formed protein crystal of lysozyme. It cannot be ruled out that the ratio of labelled to unlabelled protein is different in the crystals as compared to the bulk. In this preliminary study we did not compare this ratio for the crystal with the value in the bulk. The size of the crystals (volume 200 μm^3) was too small to investigate these crystals by our in house X-ray diffraction system. This experiment was only performed to show the incorporation of Cy-5 labelled protein into a crystal.

A clear difference between crystallising and non-crystallising lysozyme solutions was observed hours before actual crystals appeared. Therefore FCS could be a powerful tool in early screening for crystallisation conditions, since measurements are very fast (a few seconds) and can be performed shortly after mixing the components to homogeneity. The method can be easily scaled down and is capable of high throughput. Disappointingly, the difference in the behaviour of the two different proteins studied here was larger than the difference between crystallising and non-crystallising conditions, could not be explained theoretically and requires further investigation. We hoped to find a crystallisation condition with two measurements of the residence time: one very dilute (certainly non-crystallising) and one test condition (possibly crystallising). Our results show that this approach is too simple. At the present time we can only rank the conditions relative to each other. FCS can detect changes in protein-protein interaction which result in an sufficiently altered self-diffusion. It can not predict if these changes will lead to crystal formation or not, but will rule out conditions which do not alter protein behaviour and are therefore less likely to crystallise. The method is in this respect very similar to the monitoring of the osmotic second virial coefficient (George & Wilson 1994). The availability of FCS equipment is currently more limited than the availability of light scattering equipment. One major advantage of FCS is that the classification of potential crystallisation conditions can be done with simple relative measurements while for classical light scattering an absolute calibration is needed and absolute concentrations and contrast factors have to be determined for accurate results. Moreover, the high spacial resolution of FCS allows for measurements in different coexisting phases in the sample (Korlach *et al.*, 1999). FCS can become a fast and powerful tool in screening of crystallisation conditions especially as the method is still in the stage of rapid improvement (The method was recently reviewed by Krichevsky & Bonnet (2002)). A further advantage is the characterisation of each condition in a few simple numbers, which circumvents the storage problems associated with digital photography of every condition used in most current automated screening systems.

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