Heparin-aggregated RANTES can be crystallised

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Crystals of RANTES (regulated on activation normal T-cell expressed) have been grown in the presence of heparin-derived sulphated oligosaccharides which cause RANTES to aggregate severely. The crystals have a tendency to be polycrystalline but diffract to 3.8 - 1.8 Å resolution. Oligosaccharides length and stoichiometry influence aggregation, nucleation and crystal growth and quality. Surprising, rather than inhibiting crystallisation, aggregation appears to stimulate nucleation. We have co-crystallised RANTES in the presence of oligosaccharides ranging in size from 1 to 12 sugar moieties. The best crystals, both according to size and diffraction quality have been obtained with a six moiety sugar. Crystals grow in the same space group with similar cell parameters as previously reported. We find no homogeneous binding of the sulphated sugars to RANTES even after eliminating sulphate from the crystallisation conditions to avoid competition with sulphates from the sulphated sugars. There is no electron density at the sulphate positions characterised in the original structure and the residues involved in sulphate binding adopt a different side chain orientation. Either heterogeneous binding of the sulphated sugars or an active ratchet-like mechanism by which the sulphated sugars are eliminated from the crystal lattice as the crystals grow may be responsible for the absence of sugars in the structure. The fact that aggregated proteins can be crystallised is important, since it is generally accepted that proteins that are polydisperse by light dynamic scattering are poor candidates for crystallisation trials.

Keywords: aggregation; glycosaminoglycans; chemokines

1. Introduction

Aggregation is the multimeric association of molecules: dimers, trimers and larger assemblies which could reach the macroscopic scale. For crystallisation it is preferable to have a well defined multimeric state, as this would encourage the addition of well defined units to the growing crystal. Light dynamic scattering (DLS) is used to determine the degree of polydispersity within the sample, and monodisperse samples appear to have a higher probability of yielding usable crystals (D'arcy, 1994, Bergfors, 1999). On the other hand, the presence of aggregated species in an otherwise pure protein preparation is considered to be a bad omen for its successful crystallisation. Hence, to reduce the amount of wasted effort that goes into crystallisation trials with samples unlikely to yield crystals, the sample is analysed by DLS. Aggregation in proteins is believed to mediated by particular segments, often at the termini or by hydrophobic patches, and this problem can be eliminated or reduced by removing the termini and by the use of detergents of glycerol (Bergfors, 1999). Aggregation is an intrinsic property of the protein, while precipitation is induced by high protein concentrations, the addition of a precipitant, or in the case of low salt crystallisation by the removal of shielding charges.

Human RANTES belongs to the chemokine family, whose members are involved in the coordination of cellular trafficking and immune responses. Aggregation to form high molecular weight complexes is common for RANTES and other closely related chemokines (Czapleski *et al.,* 1999). The residues responsible for aggregation have been identified by mutagenesis. Among these is a glutamate residue (Glu66) at the C-terminus, and its truncation to alanine correlates with reduced aggregation (Czapleski *et al.,* 1999). At low pH, RANTES remains soluble enabling its crystallisation and its study by NMR (Skelton *et al.,* 1995). However, the addition of heparin, a polydisperse sulphated sugar polymer consisting mainly of two repeating units of 2-*o*-sulfated-α-L-idopyranosyl uronic acid (IDU) and 2-deoxy-2-sulfamido-α-D-glucopyranose-6-sulfate (SGN), or heparin fragments of six sugar moieties, or more, cause aggregation which is much more severe. Even at low pH, this high degree of aggregation renders NMR studies impossible. This aggregation is even more evident when crystallisation trials are set up. The binding of RANTES to heparin and related glycosaminoglycans (GAGs), that are part of the extracellular matrix, is related to its biological activity (Hoogewerf *et al.,*1997). These sulphated GAGs, tethered to residues on proteins of the cellular surface and extracellular matrix, are avidly bound by many endogenous proteins involved in cell adhesion, chemotaxis and signal transduction. This may be the manner by which these proteins remain localised to the cell surface.

Several protein complexes of heparin related polysaccharides have been obtained, namely, basic (Faham *et. al.,* 1996) and acidic fibroblast growth factor (Blaber *et al.,* 1996), antithrombin (Jin *et al.,* 1997) and foot-and-mouth disease (Fry *et al.,* 1999). Crystals of such complexes have been obtained either by soaking polysaccharide into pre-existing crystals as in the case of foot-and-mouth disease or by co-crystallisation. The latter is preferable since lattice contacts do not hinder backbone or sidechain movements, and the complex has more freedom of selecting a different unit cell. Since in most cases lattice contacts dominate numerically over specific protein-ligand interactions we often find, as in the case of the antithrombin complex, that despite marked differences in cell dimension, packing interactions tend to be conserved. This can be encouraged by selecting crystallisation conditions similar to those for pre-existing crystals, and allowing for a variable ligand to protein stoichiometry (Stura *et al.,* 2001).

In this paper we show that by changing the degree of aggregation through oligosaccharide reversible cross-linking of RANTES we can alter the dynamic of crystal nucleation and growth. Increased visible aggregation appears to increase nucleation while unfortunately causing crystals growth problems. Heparin induced aggregation could help in the nucleation of other GAG interacting proteins. The results presented here also help to cast doubt on the association between polydispersity and poor crystallisability.

2. Materials and methods

2.1. Crystallisation

RANTES was synthesised by solid phase peptide synthesis (Martin *et al.,* 2001). The lyophilised RANTES was re-suspended in 25 mM sodium acetate, 0.01% sodium azide, pH 4.5 to a protein concentration of 8 mg/ml. Porcine mucosal heparin was partially depolymerised with heparinase I. The enzymatic reaction was stopped by heating the digest at 100°C for 5 min. The digestion products were then size-separated using a Bio-Gel P-10 column (4.4 x 150 cm), equilibrated with 0.25 M NaCl and run at 1 ml/min. Eluted material, detected by absorbance at 232 nm, consisted of a graded series of size-uniform oligosaccharides resolved from disaccharide (dp2) to octadecasaccharide (dp18). To ensure homo-

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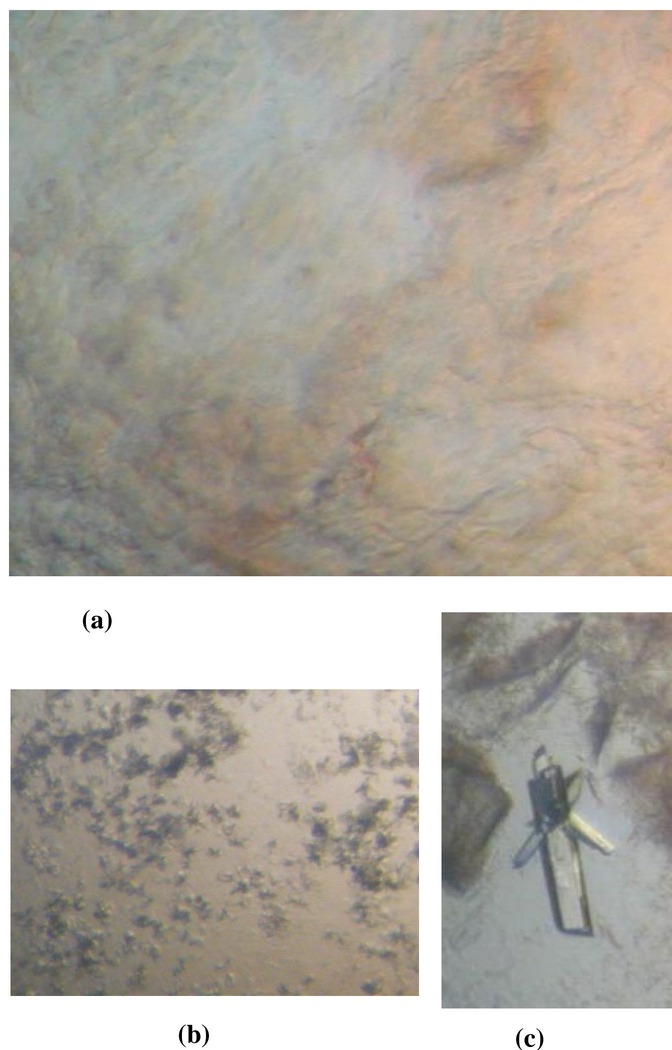

Figure 1

Aggregated RANTES and subsequent nucleation and crystal growth. (a) RANTES aggregated by the addition of dp6 hexasaccharide in the absence of precipitant. (b) Nucleation of crystals where the precipitate was. Precipitant must be added for nucleation and crystal growth. (c) The best crystals are obtained by macroseeding or streak seeding. The crystal growth clears the aggregated RANTES.

geneity, only the top fractions of each peak were pooled, and each isolated fraction was re-chromatographed on a gel filtration column to further eliminate possible contamination. Samples were finally dialysed against distilled water and freeze dried. Purified oligosaccharides corresponding to 2 repeating disaccharide subunits IDU-SGN (dp4) was re-suspended in 0.01% azide to 10 mg/ml, oligosaccharides corresponding to 3 and 4 disaccharide subunits, (dp6) and (dp8) respectively, were re-suspended to 20 mg/ml. D-glucosamine-2,6-disulfate, D-glucosamine-2-sulfate and D-glucosamine-6-disulfate were dissolved in 0.01% azide to 1 M concentration. Screening for crystallisation was carried out in sitting drop vapour diffusion using Q-plates with multiple drops per reservoir (Stura, 2001) so that the various length polysaccharides could be tested in parallel over the same precipitant reservoir. Crystals were grown by vapour diffusion at 17°C in an air conditioned room, in sitting drops. Four working solutions based on previous published conditions (Wilken, *et al.* 1999) were mixed as follows: *WS1:* 15% ethanol, 100 mM ammonium sulphate with mixed buffers consistimg of 225 mM sodium succinate, pH 4.0 and 275 mM MES pH 6.0; *WS2:* 20% PEG 4000, 6% glycerol, 0.1 M sodium acetate, 200 mM magnesium acetate, pH 4.6; *WS3:* 15% ethanol, 200 mM NaCl and mixed buffers consisting of 225 mM sodium succinate, pH 4.0 and 275 mM MES pH 6.0 and *WS4:* same as *WS3* but without ethanol. In the initial crystallisation screening the original *WS1* was used to verify that crystals could be obtained even in the presence of the aggregation caused by the sulphated oligosaccharides. Subsequently, to avoid the competition between the ammonium sulphate in *WS1* and the sulphated sugars, crystals were grown with 90% *WS3* and 10% *WS1*, thus reducing the ammonium sulphate concentration from 100 to 10 mM. Various ratios of RANTES and oligosaccharides were tested, with subsequent optimisation of ratios to improve crystal size. Drops were set up by first placing 1.2-3.6 µl of RANTES on the coverglass and then adding 0.2-1.2 µl oligosaccharides solution hence mixing the drop before adding the reservoir solution, typically the same volume as the RANTES protein solution. By varying the volumes of RANTES, the oligosaccharides solution and the reservoir solution, the stoichiometry of the oligosaccharides and the final protein concentration can also be changed. For a more complete description of the stoichiometry variation method see Stura *et al.,* (2001). Typically, crystals for data collection were enlarged using streak seeding followed by macroseeding (Stura & Wilson, 1991). The drops were seeded instantly before to prevent spontaneous nucleation. Crystal growth proceeded by a process similar to Ostwald ripening but with the frequent occurrence of crystal defects.

2.2. X-ray data collection

Data were collected for each dp2, dp4, dp6 and dp8 and dp12 for either the wild type RANTES or the less aggregating R47E-E66R double mutant. Data for the dp8-RANTES complex was collected on a Rigaku rotating anode generator running at 40kV, 120mA, with supper long mirrors on a MarResearch image-plate detector. Data were recorded at room temperature from one small crystal and processed using the HKL package (Otwinowski & Minor, 1997) (Table 2). The crystal belongs to the orthorhombic space group P212121 with cell parameters comparable to those previously reported (Table 1). The space group was assigned on the basis of the close similarity of the cell parameters to those for the uncomplexed RANTES and extinction along the three crystallographic axes. All data reported here as collected at synchrotron facilities was done under cryogenic conditions without the need of a cryosolvent as the precipitant solution is itself a cryoprotectant.

2.3. Structure determination and refinement

The 3.8 Å data set for the dp8-RANTES was used to solve the structure by molecular replacement with the program AMoRe (Navaza, 1994) using the uncomplexed RANTES dimer as the model (Table 1). The best solution obtained was in the resolution shell of 8.0 to 3.8 Å, with a strong correlation coefficient (0.67). As for the uncomplexed RANTES, there is one dimer per asymmetric unit. The solution of the molecular replacement was compared with (pdb-entry code 1eqt; 2 SO_4 sites and 1b3a; 4 SO_4 sites). The re-building of the model was limited by the poor quality of the experimental data. σA-weighted 2Fo-Fc electron density maps were calculated with the *XtalView* program (McRee, 1999) and used for model rebuilding. The refinement was carried using the programme *CNS* (Brünger *et al.*, 1998) for data at resolutions lower than 2 Å and *ARP/wARP* (Lamzin *et al.,* 1999) and *REFMAC* (Murshudov *et al.*, 1997) were used for the higher resolution data. Fo-Fc σA-weighted and un-weighted Fo-Fc electron density maps and examined with *Xfit* to determine the location of unaccounted density. The channels between

Table 1

Data collection from RANTES crystals obtained with sulphated oligosaccharides.

Data Set	Wild type with dp8	Wild type with dp6	Wild type with dp6 & dp2	Wild type with g-6,2S	R47E-E66R with dp12
Crystallisation	*WS1*	.1 *WS1* .9 *WS4*	.2 *WS3* .8 *WS4*	.2 *WS2* .8 *WS4*	.2 *WS3* .8 *WS4*
oligosaccharide	dp8: 3.3 mg/ml	dp6: 4 mg/ml	dp6: 1.3 mg/ml dp2: 2 mg/ml	60 mM g-6,2S	dp12: 3 mg/ml
A.S. concentration	100 mM	10 mM	none	none	none
Aggregation	strong	strong	medium	none	medium
Data Collection	rotating anode	LURE	LURE	ESRF-ID14EH1	ESRF-BM30
Unit cell: a, b, c (Å)	23.8, 56.9, 94.2	23.5, 56.5, 95.1	23.0, 55.9, 93.8	23.8, 56.4, 95.1	23.4, 56.8, 93.2
resolution range(Å)	20.0 - 3.8	20.0 - 2.0	20.0 - 1.96	20.0 - 1.9	20.0 - 2.3
completeness (%)	98.5	100	95.6	100	99.4
$\langle I/\sigma I \rangle$	4.0	7.4	8.9	4.5	7.5
Rsym* (%)	14.7	7.8	7.5	12.5	9.5
Refinement	not done	*ARP/wARP*	*ARP/wARP*	*ARP/wARP*	*CNS*
R-free (%)	not done	23.2	in progress†	26.2‡	in progress†
R-work (%)	not done	19.5	26.0	19.6	30.0
Sulphate positions	3 positions§	1 (weak density)	no density	no density	no density

*$Rsym = (1/N)\ \Sigma_{hkl}(1/n)\Sigma_n(\langle I \rangle - I)/\langle I \rangle$.
†refinement and rebuilding in progress, current R-free is about 4% bigger than R-work
‡ refinement and rebuilding still in progress
§ electron density is present in 2Fo-Fc σA map after molecular replacement.

the RANTES molecules in the lattice were checked using eight saccharide moieties (coordinates derived from the NMR structure of the free dodecamer: pdb-entry code 1hpn) to evaluate whether such oligosaccharides could enter of exit from the lattice without steric clashes.

3. Results and discussion

3.1. Aggregation and crystallisation results

Aggregation of RANTES in the absence of oligosaccharides has been described extensively and characterised by a variety of methods including analytical centrifugation, NMR and by size exclusion (Martin *et al.,* 2001, Czaplewski, *et al.,* 1999). The degree of aggregation induced by the addition of oligosaccharides does not allow the use of light dynamic scattering as the solution cannot be passed through a filter (Bergfors, 1999), hence the increase in aggregation can only be determined by visual inspection. Without the addition of the reservoir solution, the RANTES-oligosaccharide solution remains optically clear for sugars four subunits or shorter. On the other hand, aggregation is immediately evident as sugars six subunits or longer are added to the RANTES protein solution at 8 mg/ml independent of any addition of precipitant (Fig. 1). The rate of nucleation decreases considerably with decreasing sugar length and with decreasing number of sulphates on the monosaccharides. As expected, the aggregation caused by the longer oligosaccharides is reduced when crystallised in the presence of dp2 or ammonium sulphate. Suggesting that there may be competition for the occupation of the sulphate binding sites. For this reason the ammonium sulphate in the original crystallisation (Wilken *et al.,* 1999) was eliminated to avoid a potential competitor for sugar binding. Neither the elimination of sulphate nor the increased aggregation, that such elimination entails, prevent the nucleation and growth of crystals which adopt the same space group (Wilken *et al.,* 1999). On addition of the reservoir solution to the RANTES-oligosaccharide containing drops, either with or without visible aggregation, precipitation is induced either instantly or as a result of equilibration. The precipitant induced precipitation is morphologically different from the oligosaccharide induced aggregation.

Although the effect of the heparin derived sugars on RANTES is evident in the crystallisation and is dependent on the length of the oligosaccharides added, no homogeneous electron density is clear in any of the structures analysed so far. In the presence of 10 mM sulphate, one of the two sulphate sites remains partially occupied, no density is evident in the structures where the ammonium sulphate is replaced by sodium chloride in the presence of the sulphated oligosaccharides. In addition, the residues that are involved in sulphate binding change their side chain orientation. The sulphate positions are not replaced by the sugar sulphate. We cannot exclude the possibility of heterogeneous binding of the sulphated sugars which might be fitted in the residual electron density with an occupancy less that 0.3. Heterogeneous binding was observed in the case of foot-and-mouth disease virus (Fry *et al.* 1999), but at much higher occupancy than in our structure. However, the most surprising result is the absence of the monosaccharide, D-glucosamine-2,6-disulfate, which is present in the crystallisation at a concentration of 60 mM. With two sulphates on each saccharide, the equivalent concentration of sulphates exceeds the 100 mM present in the conditions of Wilken *et al.* (1999), suggesting that an active ratchet-like mechanism may be involved in the elimination of the sulphated sugars from the crystal lattice. The channels in the crystals are wide enough for this to take place. Such mechanism may have some parallels in the blood coagulation factors as analysed by Sinaÿ (1999).

There are minor differences between the various RANTES dimers in the asymmetric unit. Some of these differences may be caused by statistical variations in the cell parameters and in the flexibility at the termini of the protein, which have a tendency to be more mobile in all the structures. Such differences are accentuated in the R47E-E66R double mutant. Other variations affect the dimer configuration, where we observe a small rotation between the RANTES monomers in the asymmetric unit.

In the case of RANTES increased aggregation promotes increased nucleation, but slows down crystal growth and leads to inferior quality crystals. This is consistent with the overall view that aggregation is expected to affect in different ways the processes involved in crystallogenesis. The view that a high degree of polydispersity reduces the likelyhood of obtaining crystals (the threshold set arbitrarily at around 30%) should be considered with caution. It is difficult to predict how polydispersity will affect the processes of nucleation and crystal enlargement. Similarly, a monomodal distribution of particles is not predictive of a sample that will crystallise easily. However, aggregation is likely to affect in an unpredictable manner both nucleation and crystallisation. If the polydispersity observed by dynamic light scattering is an indication of the amount of misfolded protein present in the sample, the situation is more serious than if one is dealing with a thermodynamic equilibrium between different quaternary states of a macromolecular

complex. In the latter case, as in the case of RANTES, the aggregation may provide locally a high concentration of macromolecules which may rearrange to form the initial nucleus and the same process may help crystal enlargement. In the case of RANTES, seeding (both streak and macroseeding Stura & Wilson, 1991) was needed to obtain large crystals because of the large number of nuclei, poor quality of the self-nucleated crystals and a fast growth rate. Aggregation is clearly responsible for these problems which are not unique to aggregated proteins. Many macromolecular crystals often start from a semi-ordered nucleus from which polycrystals protrude (urchins). In many cases from such humble beginnings, through cycles of streak seeding and macroseeding crystals suitable for structure determination can be produced. Similarly, monodispersity, in the case of glycoproteins, is not a measure of the ease with which the protein crystals may be obtained but an indication of the difficulty that the macromolecules have in establishing protein-protein interactions because the protein surface is extensively covered with heterogeneous carbohydrates. In conclusion, aggregation is required for crystallisation, however, it is important that there should be a thermodynamic equilibrium between the various components observed in solution. The results observed with RANTES remain unusual. The aggregation is more serious than can be usually observed in proteins from which crystals can be obtained. By supposing that a biologically driven process is at work in the case of RANTES, we can explain the unexpected ease with which heparin-aggregated RANTES can be crystallised. However, to obtain X-ray quality crystals seeding is needed, hence the observation of aggregation remains an indication that crystallisation problems are likely to be encountered.

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