Investigating possible changes in protein structure during dendrimer–protein binding†

F. Chiba, G. Mann and L. J. Twyman*

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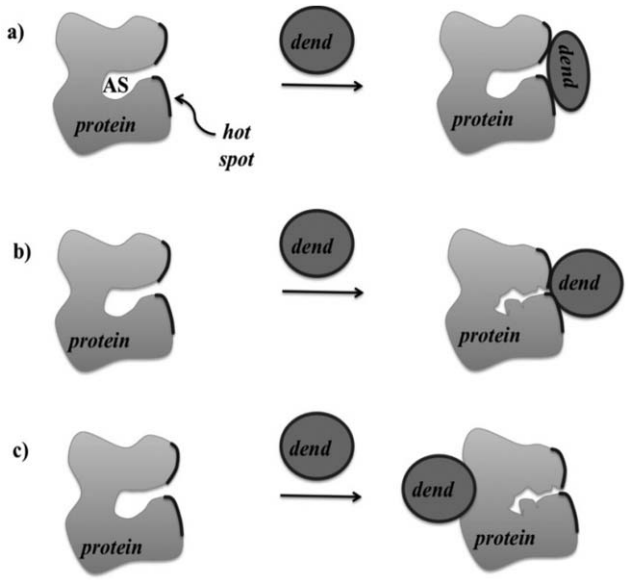
Building on our previous results that revealed a sized based mechanism for dendrimer/protein binding, the mechanism of complexation is further probed using CD spectroscopy; the results demonstrate that dendrimer/protein binding is *not* accompanied by changes in the protein's structure and that binding takes place on the interfacial area/active site entrance.

Introduction

Strategies to inhibit protein–protein interactions, as well as the design of synthetic ligands capable of binding and inhibiting proteins, are key to the development and understanding of biological processes and medicinal applications.[1–8] Within our group we have been investigating the use of dendrimers as potential inhibitors to protein/protein binding. Dendritic macromolecules are fascinating polymeric species possessing well defined structures that we and others have applied to a variety of areas.[9] Research within our group showed how polyamidoamine (PAMAM) dendrimers could be used to bind various proteins *via* a complementary size based mechanism. That is, for a particular protein, the dendrimer that bound best was the one whose size/addressable area best matched the interfacial binding area of the target proteins.[10] The proteins studied were *chymotrypsin* and the smaller protein *cytochrome-c* (which has an interfacial area less than half the size of *chymotrypsin's*). Both of these proteins have basic residues within their hot spots/interfacial area.[11] Our initial studies found that the G2.5 dendrimer **1** with 16 terminal acid groups and an addressable area of 1200 Å,^{2} bound best to the small protein *cytochrome-c*, which possesses an interfacial area of 1100 Å,^{2} whereas the G3.5 dendrimer **2** with 32 terminal acids and an addressable area of 2250 Å,^{2} bound best to *chymotrypsin*, which has a larger interfacial area of 2400 Å (Fig. 1).[2]

The active site entrance of many proteins and enzymes sits at the centre of its binding domain.[12] Therefore, when they bind a (large) ligand in this area, their active site is effectively blocked and the protein's function is impaired. This process is shown schematically in part a) of Scheme 1. The extent of this depends on the equilibrium between bound and free protein. This process of blocking the active site entrance can be used to qualitatively assesses binding. That is, the dendrimer that binds best will be the one that inhibits/blocks the best. Adopting this theory allowed us to develop our initial ideas in respect of a size based binding mechanism.[10] Nevertheless, although the results successfully proved the principle, it is ***not*** possible to conclusively identify the active site entrance as the precise location for dendrimer binding and alternatives are possible. For example, the dendrimer could bind to a remote part on the protein surface, resulting in a denaturation or a change in structure. If this occurs, then the active site and/or the active site entrance could close or become blocked, which would result in the same inhibition data being recorded. This process, is shown in part c) of Scheme 1.[13] A third possibility also exists, which is shown in b) and is effectively a combination of mechanism a) and c) shown in Scheme 1. That is, the dendrimer binds to the interfacial area as intended (blocking the active site entrance), resulting in a simultaneous denaturation of the protein. It remains unknown whether or not complex formation between the proteins and the dendrimers, altered or denatured the proteins structure. This communication describes an investigation into the effect of binding on protein structure.



Scheme 1 Schematic representing possible binding mechanisms leading to protein inhibition: a) Simple hot spot binding, where the negatively charged dendrimer binds to the positively charged hotspot region. To maximize binding the dendrimer may change shape. b) As with a), but to maximize binding the protein changes shape (denaturation). c) The dendrimer binds to a remote site resulting in denaturation and deactivation.

Results and discussion

In order to assess binding with respect to the possibility/extent of denaturation, we employed circular dichroism (CD), which is highly sensitive to polypeptide and protein secondary structure. Therefore, by comparing the spectra of the protein, to that

Chemistry Department, University of Sheffield, Sheffield, UK, S3 7HF. E-mail: l.j.twyman@Sheffield.ac.uk; Fax: +44 (0)114 2229346; Tel: +44 (0)114 2229560

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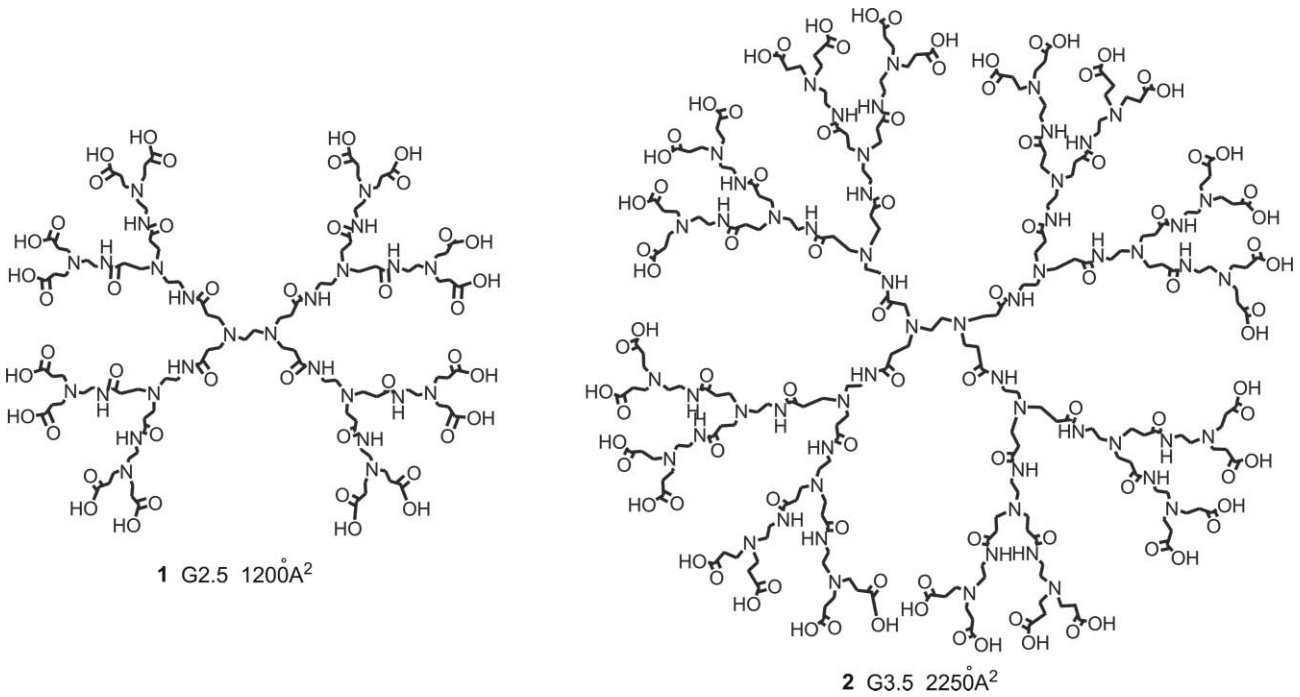

Fig. 1 The acid terminated dendrimers used in our protein binding studies. The maximum addressable area (based on the dendrimer's diameter) is shown below each generation.

obtained from the protein/dendrimer complex, would allow us to identify any structural differences. Initially we planned to measure the CD spectra of each protein in the presence and absence of its best dendrimer partner (the G2.5 and G3.5 dendrimers for *cytochrome-c* and *chymotrypsin* respectively). Therefore, solutions of *chymotrypsin* or *cytochrome-c* were made up at 1×10^{-6}M. Higher concentrations of dendrimer were used to ensure a significant level of binding was achieved. For example, the dissociation constant (Kd) for *cytochrome-c* binding to the G2.5 dendrimer is in the 10^{-4}M range. Therefore, at a similar or higher concentration the extent of binding will be greater or equal to 50%. With respect to *chymotrypsin*, inhibition experiments showed that the G3.5 dendrimer inhibited the protein by 70%, at a concentration of 1×10^{-6}M.[10] At higher concentrations significant binding must be taking place. Therefore, the final concentrations used in the CD experiments were 1×10^{-6}M in protein and 1×10^{-4}M in dendrimer. For the control and base line experiments, solutions containing just the protein (*chymotrypsin or cytochrome-c*) were made up to 1×10^{-6}M in protein.

The CD spectra of the base line/control solutions (*i.e.* just protein) were recorded first, followed by the CD spectra of the dendrimers/protein complexes. All measurements were carried out at 37 °C and at pH 7.3 (phosphate buffer, 0.1M).[14] A plot showing the CD spectra of *chymotrypsin* and its complex with an excess (1×10^{-4}M) of the G3.5 PAMAM dendrimer is shown in Fig. 2. As can be seen, the CD spectrum of the protein and the protein/dendrimer complex were identical. This confirms that binding does not denature or alter the structure of *chymotrypsin*. If the protein's structure had changed upon binding, then due to the sensitivity of the CD technique, a different series of spectra would have been obtained.[15–21] These results would suggest that the dendrimers are flexible enough to change their shape and

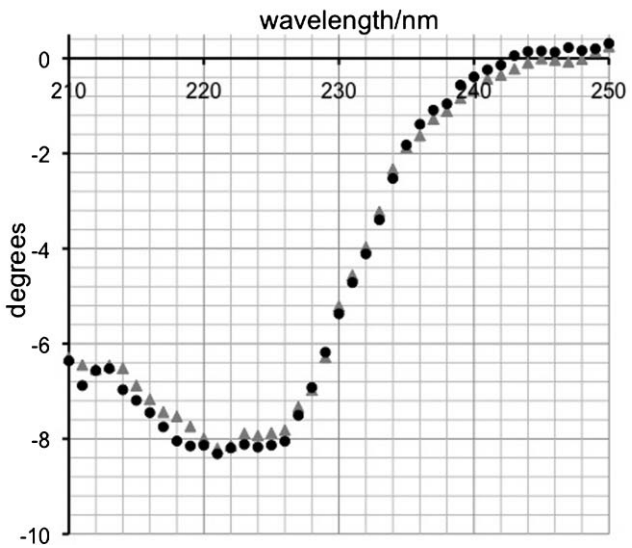

Fig. 2 The CD spectra of chymotrypsin (dark circles) and the G3.5 dendrimer/chymotrypsin complex (light triangles). Measurements recorded at 1×10^{-6}M in protein and 1×10^{-4}M in G3.5 dendrimer.

maximize binding. This leads to a minimal loss in *chymotrypsin*'s structural energy, resulting in little or no conformational change being required by the protein (to maximize the interactions – the so called induced fit mechanism). The experiment was then repeated for the smaller protein *cytochrome-c*. As before, the CD spectrum of *cytochrome-c* and its complex with an excess of the G2.5 dendrimer at 1×10^{-4}M, generated a spectrum that was identical to the native protein. Once again these results confirm that binding does not affect the structure of the protein.

If the dendrimers bound to a remote site on the protein without denaturing or affecting the conformational structure in anyway, then catalytic activity would remain unchanged. Our previous results clearly showed that this was not the case. Therefore, we can confirm that the dendrimers bind to the active site entrance (the interfacial area) and inhibit activity *via* the mechanism shown in part a) of Scheme 1.

Conclusions

In conclusion, these results show that dendrimer/protein binding does not change or affect the conformational structure or stability of the protein. As such it seems sensible to postulate that upon complex formation, structural changes to the dendrimers occur readily so as to achieve maximum binding efficiency. Therefore, based on our current and previous studies,[10] along with chemical intuition and our knowledge of protein structure (*i.e.* the propensity for higher charge densities around active site entrances and interfacial areas), we can conclude that charged dendrimers bind to a protein's interfacial area without affecting the protein's structure.

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