Dendritic Macromolecules as Inhibitors to Protein-Protein Binding

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Summary: Our initial studies into protein binding using a series of dendrimers as size selective inhibitors have been described. Two different proteins, cytochromo-c and chymotrypsin have been selected for these binding experiments.

Keywords: chymotripysin; cytochrome-c; dendrimers; protein-protein interactions

Introduction

Protein aggregates play an essential role in many biological processes; unwanted or uncontrolled interactions often result in disease.[1] Understanding the process by which proteins recognize and bind to each other is therefore fundamental to their understanding and for the development of future drugs and inhibitors.[2–6] The aim of this project was to develop and test a new methodology for the inhibition and study of protein-protein interactions. We demonstrated that protein-protein interactions could be effectively inhibited and studied if a macromolecular methodology is used in place of the more traditional small molecular approach. Although this marks a shift in medicinal chemistry paradigm, the proposed methodology precisely mimics the (selective) protein-protein binding mechanisms found in nature. It should be noted that traditional medicinal chemistry[7] has not been able to provide a ''small molecule'' solution.[8]

This is not surprising when considering the shear size of the interacting surfaces[9] can range from 500 $Å^2$ to 5000 $Å^2$. These interacting surfaces tend to be highly charged, with overall interactions dominated by simple (polyvalent) electrostatics. Although dominated by these charge-charge interactions, hydrophobic, aromatic and hydrogen bonding interactions are also vitally important with respect to selectivity.[9] This has been demonstrated by a recently study involving more than 2000 protein-protein complexes, which identified that only 3 amino acids appeared on protein hot spots[10] with a frequency greater than 10% (tryptophan, arginine an tyrosine). We must therefore consider the following parameters when designing inhibitors or probes for protein-protein binding; (i) the size of the inhibitor should match that of the proteins interfacial area (ii) the position and density of amino acids on the inhibitors surface. Hamilton and co-workers have used hydrophobic scaffold molecules possessing charged groups at their periphery.[11] Notwithstanding the considerable success of these initial systems, most of the efforts have been directed to the binding and inhibition of proteins that have relatively small interfacial areas (for example, cytochrome-c, which has an interfacial area of around 1000 $Å^2$). Ideally a series of macromolecular scaffold molecules, which are capable of interacting across a range of interfacial areas, and therefore a range of differing proteins, is desirable. Towards this end we initiated a proof of principle project aimed at determining whether or not a series of well defined polymers (known as dendrimers[12–13]) could act as efficient macromolecular inhibitors to protein-protein binding. Our target proteins were *chymotrypsin* and *cytochrome-c*, Figure 1. These were selected because they are well studied and well characterized proteins. In

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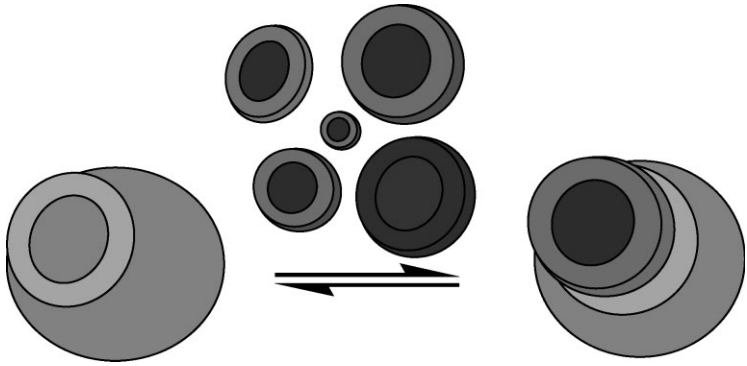

Scheme 1.
Schematic showing how proteins recognize binding partners based on "hot spot" size and functionality (i.e blue and red discs represent binding partners of differing charge).

addition, their interfacial areas differ by 100%, which makes them ideal for a "sized based" study (i.e. *chymotrypsin's* interfacial area is twice that of *chytochrome-c*).

Results and Discussion

Our approach involved the use of *charged* dendritic macromolecules. These molecules, dendrimers and hyperbranched polymers, have reasonably well controlled structures that offer a good balance between flexibility/rigidity and functionality.[14–16] Specifically we plan to use dendrimers, whose sizes increase predictably as we move from one generation to the next. The dendrimers selected were the G1.5 to G4.5 acid terminated PAMAM dendrimers possessing 8 to 64 terminal groups respectively, Figure 2. It has previously been shown that these dendrimers have relatively flexible structures, which are below the densely packed and rigid structures associated with higher generation dendrimers.[17–19] As a result the maximum surface area that each dendrimer could address can also be predicted (by measuring the diameter and squaring). Using this approach the maximum addressable areas for series of carboxylate terminated PAMAM dendrimers can be calculated. The obtained values are shown below of each dendrimer and represented schematically in Figure 2. From the obtained data it becomes apparent that the G2.5 dendrimer is closest in size to the interfacial area of the small protein cytochrome-c (interfacial area 1100 $Å^2$), and should therefore binds best. Continuing this analogy, we predicted that the larger protein, chymotrypsin (interfacial area 2400 $Å^2$), should interact best with the larger G3.5 dendrimer. To test these predictions we studied a series of PAMAM dendrimers for their ability to bind chymotrypsin[20–22] and cytochrome-c[23–27] Thus, a series of competition and inhibition experiments were undertaken to identify the best dendritic partners.[11] The results from these binding experiments showed that our predictions were correct. The dendrimer with an addressable area closest in size to the interfacial area of the particular protein bound best. Specifically for cytochrome-c, a maximum affinity was

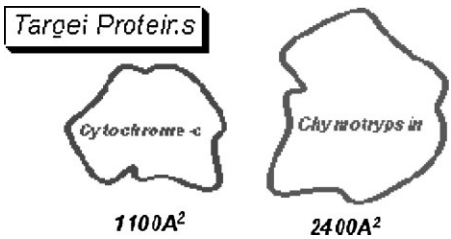

Figure 1.
Schematic showing the proteins used in our investigations. The interfacial areas are shown below each protein.

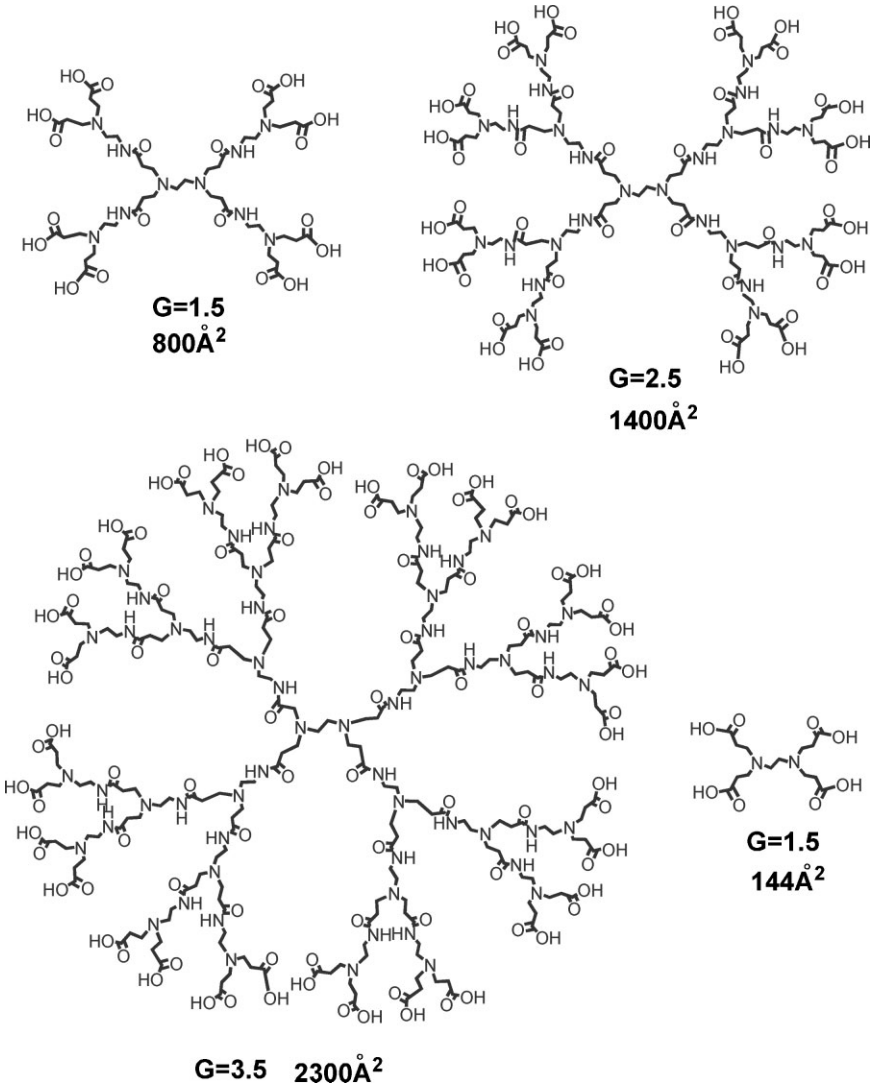

Figure 2.
The acid terminated dendrimers used for our investigations. The generation, along with their maximum addressable areas, is shown below each dendrimer respectively (for clarity, the G4.5 dendrimer with 64 terminal groups and a maximum addressable area of 3200 Å^2 is not shown). By comparing the maximum addressable area for each dendrimer, with the interfacial areas protein, we can predict that the G2.5 and G3.5 dendrimers should bind best to cytochrome-c and chymotrypsin respectively.

recorded for the G2.5 dendrimer with 16 terminal groups (results shown in Figure 3). Our inhibition/binding studies on chymotrypsin again went as predicted. The obtained results showed that the dendrimer with 32 terminal groups and a maximum addressable area of 2300 Å^2 bound best to this larger protein (results shown in Figure 4).

Conclusion

Therefore, the dendrimer that binds best has a maximum addressable area of similar dimensions to the proteins interfacial area. The preliminary results, obtained from these two proteins, support our initial hypothesis that a simple and selective binding mechanism exists based on matching

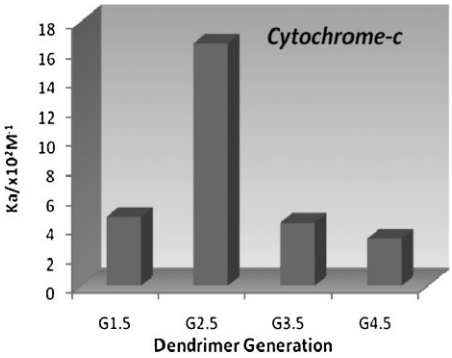

Figure 3.
Binding and inhibition data show that a dendrimer with a maximum addressable area of 1200 Å^2 binds best to the smaller protein cytochrome-c (interfacial area ~1100 Å^2).

protein and inhibitor interfacial areas. We are now extending this methodology to other proteins. We are also studying dendritic systems terminated with various amino acids to determine the relevance and importance of functional group on specific protein recognition.

Experimental Part

Chemical and Instrumentation

All chemicals and reagents were obtained from either Sigma-Aldrich or Lancaster Co. and used without further purification. Cytochrome *c* from horse heart (MW: 12384) and α-chymotrypsin type-II from bovine pancreas (MW: 25 K) were purchased from Sigma and used without further purification. The fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer (Model F-4500) and analyzed using its attached software.

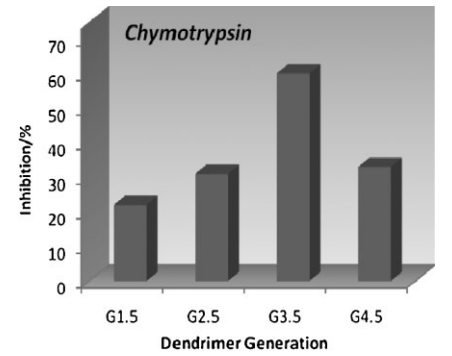

Figure 4.
Binding and inhibition data show that a dendrimer with a maximum addressable area of 2400 Å^2 binds best to the larger protein chymotrypsin, with an interfacial area ~2400 Å^2.

α-Chymotrypsin Inhibition/Binding Experiment

Unless otherwise stated all solutions were made up using a 0.1 M phosphate buffer.

Solution 1. 1.0×10^{-6} M solution of Bovine pancreatic α-chymotrypsin.
Solution 2. 1.0×10^{-6} M solution of all dendrimers.
Solution 3. 4.0×10^{-3} M solution of N-benzoyl-L-tyrosine *p*-nitroanilide (BTNA) in methanol.

The solutions required for the hydrolysis experiments were made up as follows; 10 ml of the α–chymotrypsin solution (solution 1 above) was added to 10 ml of (each) dendrimer solution (solution 2 above). The pH of the resulting solution was then checked to ensure that it remained at 7.4 (which it did in all cases). For the reaction an aliquot (2.00 ml) of this solution was added to a UV cell. 50 µl of the BTNA solution were then added (final concentrations were 1.0×10^{-4} M in BTNA, 5×10^{-7} M in dendrimer and 5×10^{-7} M in chymotrypsin). Hydrolysis was followed by monitoring product formation at 410 nm every 20 seconds using UV/Vis spectrometry. All solutions were kept at 20 °C. Initial velocities (v_o) were calculated from the linear region obtained over the first 150 seconds (Figure 1). Percentage inhibitions were calculated by taking the v_0 ratio of the control to dendrimer reaction and multiplying by 100. The number obtained is the extent of reaction (% of reaction compared to the uninhibited reaction), this was therefore converted to a percentage inhibition by subtracting it from 100. As inhibition is related to binding (i.e. binding

blocks the active site entrance), then the relative inhibitions translate to a relative binding efficiency.

Cytochrome c Binding Experiment

A tetra(carboxyphenyl)porphyrin (TCPP) stock solution of 5 μM was made up in 0.1 M phosphate buffer (pH = 7.4). The same TCPP stock solution was used to a make 5 μM cytochrome *c* solution. The PAMAM dendrimer solutions were made up to 0.01 M, also using the original TCPP stock solution (i.e. constant porphyrin concentration). For each run 2 ml of the TCPP/cytochrome-*c* solution was added to a cuvette and a fluorescence reading taken. The dendrimer solutions (10-50 μL) were then titrated in. After each addition a fluorescent emission reading was taken. The fluorimeter was set with a scan speed of 1200 nm/min with emission spectra recorded/measured between 550 and 850 nm (λ_{max} of emision ~650 nm). The excitation wavelength was set at 420 nm. Plots of saturation efficiency (saturation efficiency = (initial emission - final emission)/final emission) *vs* Log dendrimer concentration (molar) were obtained and these were fitted to a 1:1 competitive binding analysis, Figure 2 and 3.

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