Calixarene-mediated Assembly of a Small Antifungal Protein

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Abstract
Synthetic macrocycles such as calixarenes and cucurbiturils are increasingly applied as mediators of protein assembly and crystallization. The macrocycle can facilitate assembly by providing a surface on which two or more proteins bind simultaneously. Here, we explored the capacity of the sulfonato-calix[n]arene (sclxₙ) series to effect crystallization of PAF, a small, cationic antifungal protein. Co-crystallization with sclx₄, sclx₆ or sclx₈ led to high resolution crystal structures. In the absence of sclx₆, diffraction-quality crystals of PAF were not obtained. Interestingly, all three sclxₙ bound to a similar patch on PAF. The largest and most flexible variant, sclx₈, yielded a dimer of PAF. Complex formation was evident in solution via NMR and ITC experiments, with more pronounced effects with increasing macrocycle size. In agreement with the crystal structure, the ITC data suggested that sclx₈ acts as a bidentate ligand. The contributions of calixarene size / conformation to protein recognition and assembly are discussed. Finally, we suggest that the conserved binding site for anionic calixarenes implicates this region of PAF for membrane binding, a prerequisite to antifungal activity.

Synopsis
PAF, a small, cationic antifungal protein has been co-crystallized with a series of anionic calixarenes to reveal novel interaction modes. The largest ligand, sulfonato-calix[8]arene, yielded a PAF dimer in both the solid- and the solution-state.
1. Introduction

There is growing interest in the use of synthetic macrocycles as mediators of protein assembly (van Dun et al., 2017). The special case of protein crystallization (McPherson et al., 2011) has benefitted from “molecular glues” such as calixarenes and cucurbiturils that promote crystal packing (Guagnini et al., 2018; Rennie et al., 2018). The sulfonato-calix[n]arenes ( sclxₙ, Figure 1) are highly water-soluble, anionic macrocycles with diverse biosciences applications (Baldini et al. 2017; Giuliani et al., 2015; Guo & Liu, 2014). The hydrophobic core and the anionic rim of the calixarene can facilitate protein recognition, in particular, via the entrapment of arginine or lysine side chains (McGovern et al., 2012, 2014, 2015; Wang et al., 2016; Mallon et al., 2016; Rennie et al., 2017, 2018; Doolan et al., 2018; Alex et al., 2018). Consequently, sclx₄ and related compounds readily co-crystallize with the highly cationic cytochrome c and lysozyme (Alex et al., 2018; Doolan et al., 2018; McGovern et al., 2012, 2014, 2015; Rennie et al., 2017, 2018). With increasing calixarene size there tends to be more pronounced effects. For example, phosphonato-calix[6]arene (pclx₆) has ~10-fold increased affinity (with respect to sclx₄) and prompts dimerization of cytochrome c in solution (Rennie et al., 2017). Sulfonato-calix[8]arene ( sclx₈) on the other hand induces a tetramer of cytochrome c (Rennie et al., 2018). Furthermore, while calix[4]arene is locked in the bowl conformation the larger calixarenes are flexible and adopt various conformations (Figure 1) (Atwood et al., 1992; Dalgarno et al., 2003; Gutsche & Bauer, 1985; Liu et al., 2009; Perret et al., 2006; Rennie et al., 2017, 2018; Smith et al., 2006). Accordingly, sclx₄ can bind to cytochrome c either via an extended “pleated loop” or a collapsed “double cone” conformation, as evidenced by X-ray crystallography (Rennie et al., 2018).

We were motivated to characterize the sclxₙ series with a single protein and thus investigate systematically how the calixarene size and flexibility influence protein recognition and assembly. Furthermore, we were interested to study a protein for which a crystal structure was not available. Bearing in mind the tendency of sclx₄ to complex cationic proteins we chose Penicillium antifungal protein (PAF) (Marx et al., 1995, 2008) as a test case. PAF is a small (~6.2 kDa, 55 residue), lysine-rich protein (13 x Lys, pI ~9) and a potent agent against Aspergillus species and dermatophytes (Binder et al., 2010; Leiter et al., 2005; Palicz et al., 2016). The NMR structure is a twisted β-barrel composed of five antiparallel β-strands and stabilised by three disulfide bridges (Batta et al., 2009; Fizil et al., 2015, 2018). Lys30, Phe31, Lys34, Lys35 and Lys38 (loop 3) belong to a conserved region of PAF that is important for antifungal activity (Batta et al., 2009; Sonderegger et al., 2016; Garrigues et al., 2017). Similar to defensins, the mechanism of antifungal action is postulated to require interaction with anionic components on the cell membrane (Binder et al., 2010; Garrigues et al., 2017; Silva et al., 2014). Recent X-ray crystal structures have revealed how
defensin-phospholipid binding leads to oligomerization, suggesting a mechanism for membrane permeation (Poon et al., 2014; Kvansakul et al., 2016; Cools et al., 2017; Järvå et al., 2018). These observations provided further motivation to characterize PAF binding with anionic receptors.

Here, we report three PAF-sclxₙ crystal structures, demonstrating the fitness of calixarenes as crystallization agents. Interestingly, all three calixarenes bound to PAF mainly at the conserved loop 3. A similar interaction site was determined by NMR studies. These results suggest that loop 3 is favoured for recognition by anionic receptors. The largest calixarene, sclx₆, mediated a PAF dimer that was observed both crystallographically and in solution. The thermodynamics of PAF-sclxₙ interactions were characterized by isothermal titration calorimetry, providing further evidence of PAF dimerization via sclx₆. The results are discussed in the context of protein assembly and membrane binding. Finally, insights into protein complexation by flexible calixarenes are provided, including the role of PEG fragments at the protein-calixarene interface.

Figure 1. Sulfonato-calix[n]arenes. (A) Molecular structures and (B) cone (sclx₄), double partial cone (sclx₆) and double cone (sclx₈) conformations.
2. Experimental

2.1 Materials
PAF was produced as described (Batta et al., 2009; Sonderegger et al., 2016). The calixarenes were purchased from TCI Chemicals. Stock solutions of $\text{scl}_4$, $\text{scl}_6$ and $\text{scl}_8$ were prepared in water and the pH was adjusted to 6.0.

2.2 Crystallization trials
Co-crystallization experiments were performed by the hanging drop vapour diffusion method at 20°C. The reservoir solution was 20-30 % PEG 3350 and 50 mM sodium acetate, pH 5.6. A range of protein (0.7 - 7.0 mM PAF) and ligand (5 - 40 mM $\text{scl}_4$) concentrations were tested for PAF-$\text{scl}_4$ co-crystallization. Drops were prepared by combining sequentially 1 µL each of reservoir solution, protein and $\text{scl}_4$. Crystals grew at 7 mM PAF and 40 mM $\text{scl}_4$. In the case of PAF-$\text{scl}_6$ and PAF-$\text{scl}_8$ the protein-ligand solutions were premixed before combining with the reservoir solution. Co-crystals were obtained with 10 mM $\text{scl}_6$ and 40 mM $\text{scl}_8$. Crystals grew in 4-5 days ($\text{scl}_4$), 2-3 weeks ($\text{scl}_6$) or 6-8 weeks ($\text{scl}_8$).

The crystallization of ligand free PAF (7 mM) was performed with an Oryx 8 Robot (Douglas Instruments) and a sparse matrix screen (JCSG++, Jena Bioscience). Spherulites were obtained in C6 (40 % PEG 300, 100 mM potassium phosphate citrate pH 4.2) and needles grew in D7 (40 % PEG 400, 100 mM TRIS-HCl pH 8.5, 200 mM lithium sulfate). Manual crystallization trials under these conditions did not yield suitable crystals.

2.3 X-ray data collection
Crystals were cryo-protected in reservoir solution supplemented with 20 % glycerol and cryo-cooled in liquid nitrogen. Diffraction data were collected at SOLEIL synchrotron (France) to 1.30, 1.45 and 1.50 Å for PAF-$\text{scl}_4$, PAF-$\text{scl}_6$, and PAF-$\text{scl}_8$, respectively. Datasets were collected using $\phi$ scans of 0.1° over 200° (PAF-$\text{scl}_4$), 180° (PAF-$\text{scl}_6$), and 110° (PAF-$\text{scl}_8$) using an Eiger X 9M detector. In the case of pure PAF, a dataset extending to 3.0 Å was collected for the spherulites (condition C6) but was difficult to index / integrate in both XDS and iMOSFLM. The needle-like crystals (condition D7) did not diffract.

2.4 Structure determination
The observed reflections for PAF-$\text{scl}_4$ were processed with XDS (Kabsch, 2010), whereas iMOSFLM (Battye et al., 2011) was used for the PAF-$\text{scl}_6$ and PAF-$\text{scl}_8$ datasets. In all cases the data were
scaled using POINTLESS (Evans, 2011) and AIMLESS (Evans & Murshudov, 2013). Xtriage (PHENIX, Adams et al., 2010) suggested pseudo-merohedral twinning for the PAF-sclx data with twin laws -h, -k, -h-l, and estimated twin fractions of 0.025 (Britton analyses), 0.066 (H-test) and 0.022 (maximum likelihood method). The structure was determined by molecular replacement in PHASER (McCoy et al., 2007) by using the NMR structure (PDB 2mhh, conformer 1, Fizil et al., 2015) as the search model. A satisfactory solution (LLG: 134, TFZ: 7.4) was obtained with a search model in which residues 1-2, 17-24, 47-49 were deleted and all 6 cysteines were replaced by alanine. The coordinates and restraints for sclx (Ligand ID T3Y) were added in COOT. Twin refinement did not result in any significant improvement in the electron density. No twinning was indicated for the PAF-sclx6 or PAF-sclx8 data. The structures were solved by molecular replacement using the structure of PAF-sclx4 (devoid of sclx4) as the search model. The coordinates for sclx6 and sclx8 were built in JLigand (Lebedev et al., 2012). High mosaic spread (0.3-0.9) in the PAF-sclx8 dataset made it difficult to obtain better R values. Truncating the images with high mosaicity did not help in this respect. Iterative cycles of manual model building in COOT (Emsley et al., 2010) and refinement in BUSTER (Smart et al., 2012) were carried out until no further improvements in Rfree and electron density were observed. The final structures were validated with MolProbity (Chen et al., 2010) and deposited in the Protein Data Bank as PAF-sclx4 (6ha4), PAF-sclx6 (6hah) and PAF-sclx8 (6haj).

2.5 Accessible surface area (ASA) calculations
The effect of sclx4, sclx6 and sclx8 on the ASA of PAF residues in the crystal packing environments was determined in ArealMol as described previously (Alex et al., 2018).

2.6 NMR spectroscopy
The sample conditions were 0.3 or 0.5 mM 15N-PAF in 10 mM sodium phosphate buffer at pH 6.0. NMR titrations were performed at 298 K using 10-20 x 0.5-1 μL aliquots of 50 mM stocks of sclx4, sclx6 or sclx8. 1H-15N HSQC spectra were acquired with spectral widths of 12 (1H) and 19 ppm (15N) using two scans and 128 increments on a Bruker Avance-II-500 NMR spectrometer. Ligand-induced chemical shift perturbations were analysed in CCPN (Delaglio et al., 1995).

2.7 Isothermal titration calorimetry and data fitting
PAF samples were dissolved in 10 mM sodium phosphate pH 6.0. The same buffer was used to dilute stocks of sclx4 (7.1 mM, PAF 0.5 mM), sclx6 (3.6 mM, PAF 0.5 mM) and sclx8 (2.5 mM, PAF 0.3 mM) to the required concentration. Samples were degassed prior to the titration. Measurements were made at 25 °C using a Microcal ITC-200 instrument. Titrations were performed in duplicate
with similar trends between each replicate. A single replicate from each calixarene was used for model fitting. Separate titrations of each calixarene into buffer confirmed that the heats of dilution were small, exothermic and approximately constant.

NITPIC (Keller et al., 2012) was used for baseline correction and integration of the thermograms. Pytc (Duvvuri et al., 2018) was used to perform model fitting and parameter estimation. The system of equations relating the independent variables of the model (total concentrations) to the experimental observable (heat generated during injections) for the single site and the bidentate ligand models are as follows:

**Single Site Model**

\[
\begin{align*}
[P_T]_i &= [P]_i + [PL]_i \\
[L_T]_i &= [L]_i + [PL]_i \\
[PL]_i &= K [P]_i [L]_i
\end{align*}
\]

Where,
- \([P_T]_i\) is the total cell concentration of protein at the \(i^{th}\) injection (independent variable)
- \([L_T]_i\) is the total cell concentration of ligand at the \(i^{th}\) injection (independent variable)
- \(K\) is the equilibrium association constant (fit parameter)
- \(\Delta H^o\) is the enthalpy (fit parameter) associated with \(K\)
- \(V_{cell}\) is the volume of the cell
- \(V_{cell}\) is the volume of the \(i^{th}\) injection
- \(q_i\) is the heat generated from the \(i^{th}\) injection (dependent variable)
- \(q_{dil}\) is the heat of dilution (fit parameter, assumed to be constant)

\[
q_i = V_{cell} \Delta H^o ( [PL]_i - [PL]_{i-1} (1 - v_i/V_{cell})) + q_{dil}
\]

**Bidentate Ligand model**

\[
\begin{align*}
[P_T]_i &= [P]_i + [PL]_i + 2[P_2L]_i \\
[L_T]_i &= [L]_i + [PL]_i + [P_2L]_i \\
[PL]_i &= 2K_1 [P]_i [L]_i \\
[P_2L]_i &= K_1 K_2 [P]^2 [L]_i
\end{align*}
\]

\[
q_i = V_{cell} (\Delta H^o ( [PL]_i - [PL]_{i-1} (1 - v_i/V_{cell})) \\
+ (\Delta H^1_1 + \Delta H^2_2) ([P_2L]_i - [P_2L]_{i-1} (1 - v_i/V_{cell}))) + q_{dil}
\]
Where,

$K_1, K_2$ are the microscopic equilibrium association constants (fit parameters)

$\Delta H_1, \Delta H_2$ are the enthalpies (fit parameters) associated with $K_1, K_2$, respectively

The expressions for mass balance of the protein and ligand is represented by equations 1 or 4. Equations 2 or 5 define the equilibrium constants. For the bidentate ligand model equation 5 was solved numerically (Levenberg-Marquardt algorithm) to yield the free protein ($[P_i]$) and free ligand concentrations ($[L_i]$). The free concentrations were used to compute the concentrations of the other states via the equilibrium equations. The heat generated from a given injection was determined by equations 3 or 6. Parameters were constrained to physically reasonable bounds (e.g. $K_1, K_2$ between $10^2$ and $10^{10}$ M$^{-1}$) and best-fits were obtained by maximum likelihood starting from a range of initial estimates. Parameter errors and correlations were estimated using a Bayesian approach (Markov chain Monte Carlo simulations). The error for each integrated heat was determined using NITPIC (Keller et al., 2012).
3. Results and discussion

3.1 PAF-sclx₄ co-crystallization

Pure PAF proved to be recalcitrant to crystallization. A sparse matrix screen yielded spherulites or needle-like crystals only (see methods). In contrast, PAF-sclx₄ mixtures were crystallized readily from solutions containing PEG and sodium acetate. PAF-sclx₄, PAF-sclx₆ and PAF-sclx₈ co-crystals were obtained at 28-30 % PEG 3350 and 50 mM sodium acetate pH 5.6 (Figure S1, Table S1).

3.2 Data collection and model building

Datasets extending to 1.30, 1.45 and 1.50 Å resolution were collected from monoclinic (P12₁1) PAF-sclx₄, PAF-sclx₆, and hexagonal (P6₁) PAF-sclx₈ co-crystals, respectively (Table S1). The PAF-sclx₄ structure was determined by using the NMR coordinates (PDB 2mhv, conformer 1, Fizil et al., 2015) as the search model. To obtain a satisfactory solution it was necessary to delete two loops and replace all 6 cysteines with alanines. After several rounds of model building and refinement a complete PAF structure was obtained. This model was used to solve the PAF-sclx₆ and PAF-sclx₈ structures. The PAF fold and the three disulfide bridges in the X-ray structures were consistent with the NMR model (Batta et al., 2009; Fizil et al., 2015, 2018). Interestingly, the fold was altered slightly in response to sclx₄ binding (Figure S2). Superposition of the three structures revealed a Cα rmsd of 0.54 Å (PAF-sclx₆) and 0.78 Å (PAF-sclx₈) relative to PAF-sclx₄, with the largest differences at loops 2, 3 and 4. The calculated energies of the disulfide bonds (Schmidt et al., 2006) were ~3-fold lower in the X-ray structures compared to the NMR structure (Table S2).

In contrast to the PAF-sclx₄ crystals, the spherulites and needles of pure PAF failed to provide a usable dataset. The needles did not diffract and the spherulites yielded a 3.0 Å resolution dataset which proved difficult to index and integrate. The difficulty in obtaining suitable crystals of pure PAF suggests that the calixarene facilitates protein assembly and crystallization (Alex et al., 2018; Doolan et al., 2018; McGovern et al., 2012, 2014, 2015; Rennie et al., 2017, 2018).

3.3 Different calixarene, similar binding site

The asymmetric unit of the PAF-sclx₄ complexes comprised one (PAF-sclx₄ and PAF-sclx₆) or two (PAF-sclx₄) molecules of PAF. Each structure contained one calixarene, as evidenced by the 2Fo - Fc electron density maps (Figures 2 and S1). Additional electron density adjacent to sclx₄ and sclx₆ was modelled as a PEG fragment equivalent to tetraethylene glycol (EG₄) and heptaethylene glycol (EG₇), respectively (Figure 3). Sclx₄, locked in the cone conformation, encapsulated the side chain of a single lysine (Lys30), as observed previously in different protein-clx₄ complexes (Alex et al.,...
The larger, flexible \textit{sclx}_6 and \textit{sclx}_8 adopted distinct conformations and bound at least two lysines. \textit{sclx}_6 was in the double partial cone conformation (Atwood \textit{et al.}, 1992; Dalgarno \textit{et al.}, 2003), with three sulfonates pointed up and three pointed down (Figures 1B and 2B). \textit{sclx}_8 adopted the double cone conformation (Liu \textit{et al.}, 2009; Perret \textit{et al.}, 2006; Smith \textit{et al.}, 2006) with each half of the molecule acting like a calix[4]arene to bind one PAF molecule, thus mediating a crystallographic dimer (Figure 2C).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Binding site interactions in PAF-\textit{sclx}_n. (A) \textit{sclx}_4, (B) \textit{sclx}_6 and (C) \textit{sclx}_8 bind to PAF at Lys30. Note the altered conformations of Lys30 and Phe31 in each structure, while Pro29 provides a rigid hydrophobic surface for face-to-face interaction with \textit{sclx}_6 and \textit{sclx}_8. In PAF-\textit{sclx}_8, two protein chains interact with the calixarene. PEG fragments equivalent to tetraethylene glycol and heptaethylene glycol were bound to \textit{sclx}_6 and \textit{sclx}_8, respectively.}
\end{figure}

All three calixarenes bound to Lys30, while interacting also with neighbouring residues as well as other proteins (symmetry mates) in the crystal packing. Depending on the ligand size / conformation, the noncovalent contacts varied in their type and multiplicity. The PAF-\textit{sclx}_4 complex (Figure 2A) was similar to cytochrome \textit{c-sclx}_4 (McGovern \textit{et al.}, 2012), involving salt bridge and CH-\pi / cation-\pi bonds with the encapsulated lysine. Hydrogen bonds to the backbone amide NHs of Lys30, Phe31, and Asp32 were evident and the aromatic ring of Phe31 was in van der Waals contact with a \textit{sclx}_4 methylene bridge. Considering symmetry mates (Figure 4A), \textit{sclx}_4 formed substantial interfaces (>150 Å²) with 3 proteins. Interestingly, a salt bridge was formed with the N° of Ala1. Salt bridges occurred also with Lys2, Lys17, Lys22 and Lys35, emphasizing a substantial charge-charge component to complexation. In total, the protein-\textit{sclx}_4 interfaces buried ~660 Å² of protein.

\textit{sclx}_6 (1.5 times larger than \textit{sclx}_4) also completely encaged Lys30 (Figure 2B). However, one wall of the calixarene cage was composed of three phenolic groups. The phenolic oxygens were in van der Waals contact with the C°, C' and C° of Lys30, indicative of CH-\cdots O hydrogen bonding and the Lys30-N° was hydrogen bonded to a phenolic OH (rather than to a sulfonate). Other differences,
with respect to sclx₄, were water-mediated salt bridges between Lys30-N° and two sulfonates and a weak π-π interaction with Phe31 (Figure 2B). Adjacent residue Pro29 was also important for calixarene-binding (vide infra). In terms of crystal packing (Figure 4B), the larger sclx₆ was nestled between 5 proteins and formed numerous salt bridges (Lys6, Lys9, Lys11, Lys27, Lys38, Lys42). The resulting protein-ligand contacts mask ~970 Å² of protein surface. Compared to sclx₄, the more extensive interactions exhibited by sclx₆ may explain why 4 times less ligand was required to achieve crystal growth (Methods and Table S1).

The interactions of sclx₈ with PAF were similar to those observed with sclx₆ but less extensive. At twice the size of sclx₄ it might be expected that sclx₈ would mask a larger protein surface. However, sclx₈ mediated a PAF dimer (Figures 2C and 4C) resulting in total protein surface coverage of ~950 Å². The double cone conformation (compared to the “pleated loop”, Rennie et al., 2018) adopted by sclx₈ minimised its contact with protein surfaces. Salt bridge interactions involved up to 3 lysines from each monomer. Here, again a hydrogen bond was formed between the Lys30-N° and a phenolic OH. In one of the protein chains Phe31 formed an edge-to-face interaction with a sclx₈ phenolic ring. In protein chain B, Phe31 was disordered (Figure 2C).

In complex with PAF, sclx₄, sclx₆ and sclx₈ contributed an additional surface of ~550, ~850 and ~1290 Å² to the protein, respectively (calculated for a single protein). The exposed calixarene surface is a relatively homogenous “mask” that is conducive to forming noncovalent bridges with other proteins. Apparently, the calixarene acts as molecular glue (Figure 4) by providing a patch that mediates protein assembly (subsequently driving protein crystallization) in a special case of the ‘patchy particle model’ (Alex et al., 2018; Fusco et al., 2014; James et al., 2015; Staneva & Frenkel, 2015; Derewenda & Godzik, 2017).

**Figure 3. Protein-PEG-calixarene interfaces.** The protein-calixarene interfaces are completed by a PEG fragment in (A) PAF-sclx₆ and (B) PAF-sclx₈. Lys9-N° simultaneously forms ion-dipole bonds to the PEG (crown-ether like complex) and a salt bridge to one sulfonate. CH-π and lone pair-π bonds occur also between PEG and the calixarene phenolic rings.
The presence of PEG fragments (EG4 and EG7) markedly distinguished the PAF-sclx₆ and PAF-sclx₈ complexes (Figure 3). The PEG-calixarene interaction involved lone pair-π (Jain et al., 2009) and CH-π bonds, while the PEG-protein contacts included hydrogen bonds between the oxygen lone pairs and Lys9 (Lys9-Nⁱ⁻⁻O-PEG = 3.0-3.3 Å). This crown ether like Lys9-PEG interaction resembles the binding of lysine to 18-crown-6 (PDB 3wur, Lee et al., 2014). A heptaethylene glycol fragment has been observed bound to an antibody (PDB 2ajs) where it adopted a crown ether like conformation (Zhu et al., 2006), compared to the extended conformation in PAF-sclx₆. And a crystal structure of a SH3 domain (PDB 5xg9) revealed various PEG fragments at protein-protein interfaces (Gautam et al., 2017). These examples suggest the role of PEG as interface “filler”. Perhaps the PEG fragments (Figure 3) contribute towards calixarene conformation selection / stability.

Figure 4. Calixarenes as molecular glues. The crystal packing is dominated by PAF-sclx₆ interactions in (A) PAF-sclx₄, (B) PAF-sclx₆, and (C) PAF-sclx₈. This observation suggests that the calixarene is a molecular glue for protein assembly. Proteins, calixarenes and unit cell axes are depicted in grey, green and blue, respectively. The PEG fragments are depicted as sticks.
3.4 Selectivity of PAF-sclxₙ complexation, why Lys30?

Considering that PAF contains 13 lysines the question arises why Lys30 was selected by sclxₙ. Accessible surface area (ASA) calculations were used to probe the selectivity of sclxₙ for the Pro29-Lys30-Phe31 patch over other possible binding sites (Figure 4). The calculations accounted for contributions from symmetry mates in the crystal packing (Alex et al., 2018). The effect of ligand binding on the ASA of all Lys, Pro, Phe and Tyr residues is plotted in Figure 5. At least half of the lysines, including Lys30, are highly exposed (ASA ≥ 125 Å²) in each structure in the absence of sclxₙ. This observation suggests that steric accessibility (McGovern et al., 2014) was not the determining factor in sclxₙ selectivity. For example, Lys2 (>150 Å²) was significantly masked (ΔASA ≥ 15 %) by binding with sclx₄ only. Perhaps a salt bridge interaction with Asp46 reduced the availability of Lys2. In contrast, Lys30 was strongly effected by all 3 calixarenes (ΔASA up to 80 %). Adjacent residue Lys27 was also strongly effected in the complexes with sclx₆ and sclx₈. The differences in the degree of masking can be attributed to the calixarene sizes (small sclx₄) and conformations (“double cone” sclx₈). However, sclx₆ had more in common with sclx₆ than sclx₄. For example, Lys9, Lys11 and Lys38 were 30-50 % buried by sclx₆ or sclx₈ while sclx₄ had no effect on these residues. Overall, calixarene binding resulted in significant masking of 5 (sclx₄), 8 (sclx₆) and 6 (sclx₈) lysines.

PAF has five aromatic residues, Phe25, Phe31, Tyr3, Tyr16 and Tyr48 (Figure 5). The latter is highly solvent exposed (~200 Å²) and might be expected to interact with sclxₙ. However, only minor contributions were evident (Figure S3). Phe31 was the dominant aromatic residue for sclxₙ complexation. The adjacent Lys30, Lys34 and Lys35 may facilitate (via charge-charge interactions) calixarene binding here, compared to Tyr48, which is proximal to Lys2 only. The contribution of Pro29 merits special attention as it completes the binding site for both sclx₆ and sclx₈, via face-to-face hydrophobic stacks with a phenolic ring (Figure 2B and 2C). These interactions are reminiscent of polyphenol binding to proline-rich proteins (Baxter et al., 1997; Charlton et al., 2002; Quideau et al., 2011). The rigid pyrrolidine ring appears to provide a stable platform for binding the “floppy” sclx₆ or sclx₈. Thus, it is perhaps unsurprising that the only proline residue in PAF was involved at the binding site.

As such, it appears to be the combination of the Pro29-Lys30-Phe31 motif and adjacent lysines (charge-charge interactions) that stabilize sclxₙ binding and impart selectivity. This region has been implicated in PAF function, with decreased antifungal activity when Phe31, Lys35 or Lys38 were mutated to Asn or Ala (Batta et al., 2009; Sorderegger et al., 2016; Garrigues et al., 2017). The selectivity of the anionic calixarenes for this site suggests that it may be involved in cell membrane binding and permeation as required for antifungal activity.
Figure 5. Accessible surface area (ASA) plots. Accessibility of Lys, Pro, Phe and Tyr residues in ligand-free (black) and bound (grey) PAF. The PAF-sclx₄ data correspond to chain A.

3.5 NMR Characterization and Comparison with the Solid State

PAF-calixarene binding in solution was assessed by NMR spectroscopy. Titrations were performed by the addition of microliter aliquots of sclx₄ to ¹⁵N-labelled PAF, which was monitored by ¹H-¹⁵N HSQC spectroscopy (Fizil, et al., 2018; McGovern et al., 2012). The overlaid spectra (Figure 6) revealed increasing chemical shift perturbations (Δδ) as a function of sclx₄ or sclx₆ concentration, indicative of fast to intermediate exchange between the ligand-free and -bound states. Some biphasic shifts were evident for sclx₆. Severe broadening effects were observed with ≥ 0.3 eq sclx₈ indicative of a slow-exchange process and suggesting the possibility of ligand-mediated oligomerization (Doolan et al., 2018; Fonseca-Ornelas et al., 2017; Mallon et al., 2016; Rennie et al., 2017, 2018).

The Δδ plot (Figure 6) shows a clear selectivity for sclx₄ binding to Lys30 and neighbouring residues 31-36. In the crystal structure, all of these residues occurred in the vicinity of sclx₄. Significant Δδ were observed also for the C-terminal Val52 and Cys54, which are further from the crystallographic binding site. However, both of these residues are adjacent to Pro29, and Cys54 is hydrogen bonded to Lys34, suggesting a mechanism for how these resonances sense ligand binding. In the presence of sclx₆ the Δδ plot again shows a preference for binding around Lys30 as well as effects at the C-terminus (Val52-Nα is hydrogen bonded to sclx₄). However, compared to
The shifts are 2-4 times larger and other segments of the primary structure (residues 6-13 and 42-45) were affected also. These two regions correspond to additional sclx₆ binding sites evident in the crystal packing. Therefore, the NMR data suggests that the PAF-sclx₄ interaction is fluxional, with the calixarene exploring different patches on the protein surface, as observed previously for cytochrome c - sclx₄ complexes (Doolan et al., 2018; McGovern et al., 2012). Judging from the magnitude of the shifts, binding to Lys30 is preferred while a weaker interaction occurred at a patch involving Lys6 and Lys42.

**Figure 6. NMR characterization of PAF - sclx₈ complexation.** (A) Region from overlaid $^1$H-$^{15}$N HSQC spectra of pure PAF (black contours) and in the presence of 0.1-0.6 mM ligand (coloured scale). Biphasic shifts occurred for resonances K30, K34 and C36 in the presence of sclx₈. Resonances K11, C28, K30, K34, and C36 were broadened at 0.3 mM sclx₈ while resonances T8, K11, D32, T37 were broadened beyond detection at 0.6 mM sclx₈. (B) Plots of chemical shift perturbations measured for PAF backbone amides in the presence of 0.6 mM sclx₄, sclx₆ or sclx₈. Blanks correspond to Pro30 and undetectable resonances (due to broadening).
The titrations with \textit{sclx}_8 resulted in yet different effects. In addition to pronounced perturbations of Lys30 and neighbours, substantial broadening effects occurred. Cys28, Lys30, Lys34 and Cys36 broadened at 0.3 mM, and Thr8, Lys11, Asp32 and Thr37 broadened beyond detection at 0.6 mM \textit{sclx}_8. These 8 residues are located at the crystallographically-defined binding site. Thus, the broadening effects may be indicative of PAF dimerization, consistent with the \textit{sclx}_8-mediated dimer in the crystal structure (Figure 2C). Previously, we observed a complete loss of the HSQC spectrum of cytochrome c in complex with \textit{pclx}_6, which also yielded a dimer in the solid state (Rennie \textit{et al.}, 2017).

### 3.6 Thermodynamics of PAF-sclx\textsubscript{n} complexation

Isothermal titration calorimetry was used to characterize the PAF-sclx\textsubscript{n} binding affinities and stoichiometries (Figure 7). The data were fit to a single site or a bidentate ligand model. The latter model describes a bidentate ligand that can bind two protein molecules and was necessary to describe the obviously biphasic data for \textit{sclx}_8. The choice of this model is supported by the observation of a PAF-sclx\textsubscript{n}-PAF dimer in the crystal structure, and by the spectral broadening in the NMR experiments. All of the fit parameters were well-determined by the data (Table 1), with parameter errors assessed by Bayesian methods (Patil \textit{et al.}, 2010).

![Figure 7. ITC analysis of PAF - sclx\textsubscript{n} complexation.](image)

The isotherms for \textit{sclx}_4 injected into PAF were fit to a single site binding model with $K_d \sim 110 \mu$M. In contrast, the isotherms for \textit{sclx}_8 were biphasic (Brautigam, 2015) and fit to a bidentate ligand
model with $K_d$ of $\sim 10$ and $\sim 30$ µM, for binding the first and second molecule of PAF, respectively. The isotherms for sclx₆ were intermediate between sclx₄ and sclx₈, suggesting that this ligand may exhibit weak bidentate binding. A satisfactory fit for this data was not obtained with either model. The ITC data demonstrate an increasing affinity for PAF as calixarene size increases and a switch in binding mode from the small, rigid sclx₄ (single site) to the large, flexible sclx₈ (bidentate).

**Table 1.** Thermodynamic of PAF-sclxₙ complexation determined by ITC.*

<table>
<thead>
<tr>
<th>[Ligand]ᵃ</th>
<th>[PAF]ᵇ</th>
<th>$K_d$ (µM)</th>
<th>ΔH (kJ/mol)</th>
<th>TΔS (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7143 (1248)</td>
<td>500 (412)</td>
<td>107 (0.0, 0.0)</td>
<td>-16.9 (0.1, 0.1)</td>
<td>-5.6 (0.2, 0.2)</td>
</tr>
<tr>
<td>3623 (633)</td>
<td>500 (412)</td>
<td>15.4 (0.0, 0.0)</td>
<td>-28.2 (0.2, 0.1)</td>
<td>-0.7 (0.2, 0.2)</td>
</tr>
<tr>
<td>3623 (633)</td>
<td>500 (412)</td>
<td>47.8 (0.0, 0.0)</td>
<td>-9.2 (0.1, 0.2)</td>
<td>-15.4 (0.4, 0.3)</td>
</tr>
<tr>
<td>3623 (633)</td>
<td>500 (412)</td>
<td>45.8 (3.5, 4.1)</td>
<td>-20.1 (0.4, 0.4)</td>
<td>-4.6 (0.6, 0.6)</td>
</tr>
<tr>
<td>2500 (437)</td>
<td>300 (247)</td>
<td>10.6 (1.3, 1.4)</td>
<td>-3.5 (0.3, 0.3)</td>
<td>-24.8 (0.3, 0.3)</td>
</tr>
<tr>
<td>33.5 (4.8, 6.5)</td>
<td>36.0 (1.7, 1.4)</td>
<td>10.5 (1.8, 2.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fit values are median (2.5% quantile, 97.5% quantile) from Markov chain Monte Carlo. In the case of sclx₆ the fit parameters for both models are shown.

ᵃCalixarene concentrations in the syringe, final concentration indicated in parenthesis.

ᵇProtein concentration in the cell, final concentration indicated in parenthesis.
4. Conclusions
Using a combination of X-ray crystallography and NMR spectroscopy it was demonstrated that the sclxn series binds selectively to the highly cationic PAF. Despite the varying size and conformational flexibility, sclx4, sclx6 and sclx8 bound similarly the Pro29-Lys30-Phe31 motif in loop 3. The selectivity of the anionic calixarenes for this motif, and the role of loop 3 in antifungal activity, suggests that this region may be required for membrane binding. In addition to charge-charge interactions (evidenced by numerous lysine to sulfonate salt bridges) other noncovalent bonds including CH-π and π-π (via Pro29 and Phe31, respectively) participated in ligand stabilization.

The presence of PEG fragments at the protein-sclx6 and -sclx8 interfaces suggests that PEG acts as a “filler” to complete the binding site, potentially reinforcing the calixarene conformation.

The structures of all three sclxn - PAF co-crystals highlight the potential of calixarenes as a ‘sticky patch’ on the protein surface that facilitates assembly and crystallization. In the case of the sclx4 and sclx6 co-crystals (P12,1) it is evident that the calixarene is a dominant contributor to the crystal packing (Figure 4). Similarly in the sclx6 structure (P6) the packing involves substantial protein-calixarene contacts, and the structure is further interesting as sclx6 mediates a PAF dimer. Previously, we found that sclx8 mediates a tetramer of cytochrome c (Rennie et al., 2018). Generally, it seems that calixarene-mediated protein crystallization may be a special case of the patchy particle model for protein assembly (Alex et al., 2018; Fusco et al., 2014; James et al., 2015; Staneva & Frenkel, 2015; Derewenda & Godzik, 2017). Considering that PAF alone did not yield diffraction-quality crystals, we conclude that co-crystallization with sclx6 was beneficial. Anionic calixarenes may generally facilitate crystallization and structure determination of small cationic proteins.

The binding surfaces observed in the NMR experiments were consistent with the X-ray data. However, the NMR effects were more pronounced with increasing calixarene size suggesting that the larger calixarenes mask a greater portion of the protein surface and / or lead to assembly in solution. Similarly, the ITC experiments revealed tighter affinities and more complex effects with increasing calixarene size. In particular, sclx4 behaved as a bidentate ligand that facilitated PAF dimerization. These data add to the growing evidence of calixarene-mediated protein assembly in solution (Doolan et al., 2018; Rennie et al., 2017, 2018). In terms of the biological relevance of these data it is noted that defensin oligomerization (upon phospholipid binding) has implications for antifungal activity (Poon et al., 2014; Järvå et al., 2018). Perhaps calixarenes can be used to modulate the activity of PAF and related proteins.
Acknowledgements

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References


