The SilE protein is suspected to have a prominent role in Ag⁺ detoxification of silver resistant bacteria. Using model peptides, we elucidated both qualitative and quantitative aspects of the Ag⁺-induced α-helical structuring role of His- and Met-rich sequences of SilE, improving our understanding of its function within the Sil system.

The bactericidal power of Ag⁺ has been exploited for hundreds of years, and recent efforts have been made to develop new silver-based compounds able to tackle multidrug-resistant bacteria.1,2 However, several Gram-negative bacteria are able to survive in silver contaminated media, thanks to intrinsic and/or acquired HME-RND (Heavy-Metal-Eﬄux Resistance-Nodulation-Division) eﬄux pumps.3,4 The latter are the most common bacterial defence against toxic metal ions.5 The Cus and Sil systems are part of the HME-RND family and have shown the capacity to make bacteria silver resistant.6-9 While the Cus system is expressed in the context of chromosomal mutations, the Sil system is plasmid encoded. Besides the fact that they can be horizontally transferred, resistance plasmids have relatively low ﬁtness costs in comparison with chromosomal resistance mutations.10 As a consequence, plasmid-encoded resistances are deemed to be more widespread than mutational resistances.11 Hence, the full understanding of the Sil system is of prime importance in the context of ﬁghting silver resistant bacteria. Both systems function along similar lines and possess a common basis (CusCFBA and SilCFBA). However, this basis is not enough to provide a signiﬁcant silver tolerance, and needs to be complemented by a control of the cellular silver concentration.

Therefore, the CusCFBA transporter is associated to a deﬁciency in OmpC and OmpF porins, and the SilCFBA transporter is associated to three other proteins, SilP, SilE and SilG, of which only SilE is mandatory to confer the resistance.7 Moreover, Randall et al. have shown that SilE could substitute the need for porin loss associated with the Cus system.7 All this points out the prominent role of SilE in the bacterial resistance to Ag⁺. Nevertheless, the structure of the latter has never been solved and its mode of action is still debated. Previous studies have proposed histidine and methionine residues to be engaged in the coordination of Ag⁺ ions by SilE via eight MX₂H and HX₃M motifs and one HX₅M motif, all individually able to bind a single Ag⁺ ion with Kₐ in the μM range.12-14 However, the involvement of these motifs in Ag⁺ coordination in the full-length SilE protein has never been proved, and Ag⁺/SilE binding aﬃnities have not been reported to date. The mechanistic aspects of the Ag⁺-induced folding of the protein and its structural organization remained also unclear, making the understanding of its structure/function relationship diﬃcult. Here we report qualitative and quantitative aspects of the Ag⁺ coordination by structural motifs of SilE, through the characterization of the silver binding sites in terms of coordination numbers and geometries, binding aﬃnities, and Ag⁺-induced structural folding. Based on our studies, the ﬁrst structures of SilE key sequences have been solved and a non-cooperative Ag⁺ binding is proposed for SilE.

The eight MX₂H and HX₃M motifs of SilE form two types of “twin” sequences, namely MX₂H₄HX₅M (type A) and HX₄MX₂HX₃M (type B) (Fig. 1). Model peptides of these four sequences have been synthesized as N-terminal acetylated and C-terminal amidated peptides. The B1 motif has been additionally studied as 14-amino acid peptide (B1b), to investigate the potential involvement of Met91 in Ag⁺ coordination, and a sixth model peptide corresponding to the 28 C-terminal amino acids of SilE (B2b) has also been studied to examine the Ag⁺ coordination of a model peptide containing three HX₃M motifs. The different peptides were investigated by ¹H NMR titrations to determine their Ag⁺-binding capacity. The ¹H chemical shifts of both His and both Met residues (His-H² and His-H³) are...
significantly affected by the addition of Ag⁺, indicating their involvement in Ag⁺ coordination. The evolution of the chemical shift is clearly in agreement with the binding of two Ag⁺ ions per peptide with a plateau after 2 equiv. of Ag⁺ added (Fig. 2a and Fig. S1, ESI†). Similarly, the B2b model that harbors three HX₂M motifs can bind up to three silver ions (Fig. S1, ESI†). The behavior of B1b, which displays two His and three Met, is more complicated. It is described in detail in the ESI† (Fig. S1 and S2). This peptide can bind three Ag⁺ ions, the third of which has a lower affinity (mM range vs. μM, see below and ESI†). The monitoring of the chemical shifts reveals that the HX₂M motifs are the two primary binding sites. The third Ag⁺ ion may bind to Met90 and Met91. For all peptides, DOSY experiments were recorded in the presence or absence of Ag⁺ ions (Fig. S3–S8, ESI†) in order to compare the diffusion coefficients of the apo-peptides and their silver complexes. They indicate species of similar size suggesting the formation of silver complex species involving a single peptide, i.e. Ag₃P or Ag₅P. Thus, we can conclude that all these peptides bind one Ag⁺ ion per MX₂H or HX₂M motif. From previous studies on HX₂M and MX₂H tetrapeptides, we can infer a His–Ag–His and Met–Ag–Met coordination mode cannot be ruled out. Further structural details about the Ag⁺/SilE interaction arise from circular dichroism (CD) and NMR. The two types of sequences (MX₂Hₓ₂Hₓ₆Hₓ₂M, A and HX₂MX₂Hₓ₂M, B), which exhibit random-coil conformations in their apo-form, adopt different structures in the presence of silver ions. CD experiments reveal the appearance of two minima at 207 nm and 223 nm for type B peptides, clearly indicating that they are folding into z-helices upon Ag⁺ addition (Fig. 2b), in contrast to type A peptides, which exhibit a poor z-helix signature evolution during Ag⁺ complexation and appear thus less structured in their holo-form (Fig. S9, ESI†).15

To gain deeper understanding of the coordination geometries and the structural features of the silver binding sequences of SilE, the NMR solution structures of the different Ag⁺/SilE-derived peptide complexes have been solved (Fig. 3). As expected, the Ag⁺ binding to B1 and B2 induces a well-defined helical structure of the respective model peptides. On the other hand, A1 and A2 remain partly unstructured. In both cases, however, the orientation of His and Met side chains, which are alternating on the same side of the z-helix in type B peptides, clearly indicates that each Ag⁺ ion is bound to one HX₂M or MX₂H motif, excluding His–Ag–His or Met–Ag–Met coordination geometries. Therefore, the 1:1 Ag⁺/HX₂M or MX₂H stoichiometry pointed out by both tetra- and poly-peptide model studies suggests a linear His–Ag–Met coordination mode, which is common for Ag⁺ ions. Moreover, B1b and B2b peptides adopt an z-helical fold upon Ag⁺ binding similar to those of B1 and B2, respectively, and their structures overlap to a large extent (Fig. S12, ESI†). In the case of B1b, the potential coordination of a third Ag⁺ ion by Met90 and Met91 (see above and ESI†) does not influence the folding of B1b. In the case of B2b, no NOE between the

Fig. 2 B1/Ag⁺ NMR and CD titrations. (a) Histidine (His-H² and His-H⁶) and methionine (Met-H⁴) 1H resonances shift by addition of AgClO₄ (0 to 4 mM) to a solution of B1 (1 mM) in HEPES buffer (20 mM, pH 7.8). The insert depicts the 2:1 stoichiometry of the Ag⁺B1 complex. (b) z-Helix folding of B1 (10 μM, in NH₄Ac 1 mM, pH 7.4) by addition of AgClO₄ (0 to 48 μM) evidenced by the increase of the intensity at 223 nm (left). Plot of the CD signal at 199 nm (square) and 223 nm (circle).
N-terminal HEFM motif and the two C-terminal motifs composing the B2 sequence are observed, indicating that they bind different Ag⁺ ions.

As previously shown, isolated MX₂H and HX₂M motifs intrinsically bind silver ions with moderately strong affinities (log K_{ass} = 5.3–6.6). However, it has to be determined whether the proximity of the binding sites and the Ag⁺-induced z-helix folding of the backbone affect the silver binding affinities of the various MX₂H or HX₂M motifs. Fluorescence competition titrations were chosen to determine the binding constants of the model peptides, requiring the design of a fluorescent probe. Inspired by the MX₂H and HX₂M motifs of SilE, a tryptophan containing tetrapeptide (HEWM) has been synthesized for this purpose, and its ability to bind Ag⁺ has been investigated (Fig. 4). A fluorescence titration of HEWM by Ag⁺ in HEPES buffer shows the formation of a 1:1 complex with a 50% quenching of the tryptophan emission upon Ag⁺ binding. ²H NMR confirms the stoichiometry of the complex and the binding of both histidine and methionine to Ag⁺ (Fig. S13, ESizontally). Based on the difference of fluorescence intensity between the apo- and holo-forms of the probe, competition experiments were performed with four different tetrapeptide competitors of known Ag⁺ affinity (MDQH, MNEH, HEFM and HQAM)¹³ in order to determine the association constant of the probe (K_{ass} = [AgP]/[Ag][P]), yielding log K_{ass} = 6.4 ± 0.2 (Fig. 4, Fig. S14–S16 and Table S3, ESizontally).¹⁶ Then, in order to determine the binding affinities of A and B peptides, similar experiments were performed with our six SilE-derived peptides using HEWM as competitive fluorescent probe (Fig. S17–S20 and Table S4, ESynchronously). The resulting binding constants are in the same order of magnitude as those obtained for the corresponding tetrapeptide complexes. For instance, the peptide A₁ binds two Ag⁺ ions (K₁ = [AgP]/[Ag][P] and K₂ = [AgP]/[Ag][AgP]) with log K₁ = 6.6 ± 0.3 and log K₂ = 5.6 ± 0.4, while the two individual motifs composing the sequence, HETM and MDQH, bind one Ag⁺ with log K₁ = 6.4 ± 0.1 and 5.8 ± 0.1, respectively, when studied as tetrapeptides (Table 1). However, affinity constants of the trimetallic Ag⁺B₁b and Ag⁺B₂b complexes could not be extracted with this method, since it ends up with a too large standard deviation on the K₁ value. Overall, with stepwise association constants (log K₁ and log K₂) between 5.1 and 6.7 (±0.5), the affinities of the herein described models for the two silver ions are in the same range as the intrinsic affinities of the MX₂H and HX₂M sites (log K_{ass} = 5.3–6.6).¹³ Therefore, these similarities confirm the His–Ag–Met coordination mode in type A and B peptides. Moreover, the binding affinities of B₁ and B₂ being similar to those of A₁ and A₂, no significant effect of the peptide structuration has been observed. Obviously, the presence of two silver binding sites in the model and the Ag⁺-induced z-helix folding of the peptide do not have a significant effect on the silver binding constants. Therefore, the four MX₂HX₂M and HX₂MX₂HX₂M sequences found in SilE are proposed to bind Ag⁺ in a non-cooperative binding mode.

In the context of accurate characterization of protein–metal interactions, the use of model peptides can help to reach qualitative and quantitative insights, which are not necessarily attainable by working with an entire protein. In this instance, while several research groups have investigated the role of SilE for two decades, no structural or numerical characterization of the metal centers has arisen. In contrast, the herein described work not only provides the structure of the four silver binding sequences of SilE, but also quantises the silver docking. As suggested by previous studies on MX₂H and HX₂M ultrashort model peptides of SilE, the latter seems to bind up to 8 Ag⁺ ions via its MX₂HX₂HX₂M and HX₂MX₂HX₂M sequences, each of them binding two Ag⁺ ions. A ninth Ag⁺ ion could nevertheless be bound to the isolated HXM motif (H38–M40) with a similar affinity.¹³ Asiani et al. suggested a complete folding of SilE after the binding of 6 Ag⁺ ions (out of a total of 8 Ag⁺ ions bound), and the presence of two core motifs (A₇₇–M₉₁ and E₁₁₀–F₁₂₀) which, when folded into z-helices, should facilitate the folding of the rest of the protein.¹⁷ The different backbone foldings between type A and type B sequences upon Ag⁺ complexation support this hypothesis. However, while the first core motif corresponds to the herein described B₁ peptide,
which folds into a stable $\alpha$-helical structure upon Ag$^+$ binding, the second core motif is a truncated version of the A2 peptide, which does not adopt a well folded secondary structure in the presence of Ag$^+$. A further motif is identified, which clearly suggests the formation of His–Ag–Met motifs with linear coordination, the structure of which is common in Ag$^+$ coordination chemistry. In order to assess if these peptides can accommodate linear His–Ag–Met coordination, the structure of B2 was calculated once again using NMR-derived distance and dihedral angle restraints but forcing a linear helix geometry. The obtained structure retains the helical fold of B2 and displays no violation of NOE and dihedral constraints. It clearly establishes that this linear His–N–Ag–S–Met geometry is indeed possible within a HX$_2$M motif as part of an $\alpha$-helix (Fig. 5).

The plots of NMR and CD titrations of the herein described model peptides by Ag$^+$ always adopt hyperbolic binding curves, suggesting a non-cooperative binding of Ag$^+$ ions by the different binding sequences of SilE. Numerical values of stepwise affinity constants of the Ag$^+$/SilE-derived peptide complexes ($K_1 < 6.7 \pm 0.4$ and $K_2 < 5.6 \pm 0.4$) support this hypothesis. Most surprising, however, is that type A and type B peptides bind Ag$^+$ with similar affinities, and that $K_1$ and $K_2$ are in the same range for all models, implying that the structure of type B peptides is neither beneficial nor detrimental to silver binding in comparison to the shorter HX$_2$M and MX$_2$H peptides. Indeed, the range of affinities is consistent with the previous hypothesis that SilE could buffer silver ions in case of high Ag$^+$ overload, avoiding the saturation of the periplasmic adaptor SilB, in charge of the Ag$^+$ externalization. Moreover, while the interaction between Ag$^+$ ions and the sensor kinase SilS has not been investigated to date, qualitative and quantitative data are available for its homologue CusS. This homodimeric protein possesses two different silver binding sites, of which one is more important for function, and is conserved between CusS and SilS. The complexation of the four Ag$^+$ ions by CusS, which is also mainly carried out by His and Met residues, is governed by an apparent $K_d$ in the $\mu$M range. Therefore, we propose that SilE could regulate the free Ag$^+$ periplasmic concentration at a level to which enough Ag$^+$ ions remain available to continuously depress the expression of the silCFBAGP operon via SilRS, but not enough to overload the silB operon.

In conclusion, by means of model peptides, we qualitatively and quantitatively characterized the interaction between the different binding sequences of SilE and Ag$^+$ ions. This study provides the first solution structures of the different silver centers found in SilE. When compared to other components of the Sil system, the characterization of the strength of Ag$^+$/SilE interactions supports the hypothesis that SilE buffers Ag$^+$ ions in the $\mu$M range, and hence, sustains the Ag$^+$ export and the silCFBAGP operon expression. In view of the different metal centers in the Sil and Cus systems, it is very likely that bacteria control the metal ion transfer between the different partners of the efflux systems with the number of histidine and methionine residues involved in metal coordination.

**Conflicts of interest**

There are no conflicts to declare.

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