Iron Complexes

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An $[\{Fe(mecam)\}_2]^{6-}$ Bridge in the Crystal Structure of a Ferric Enterobactin Binding Protein**

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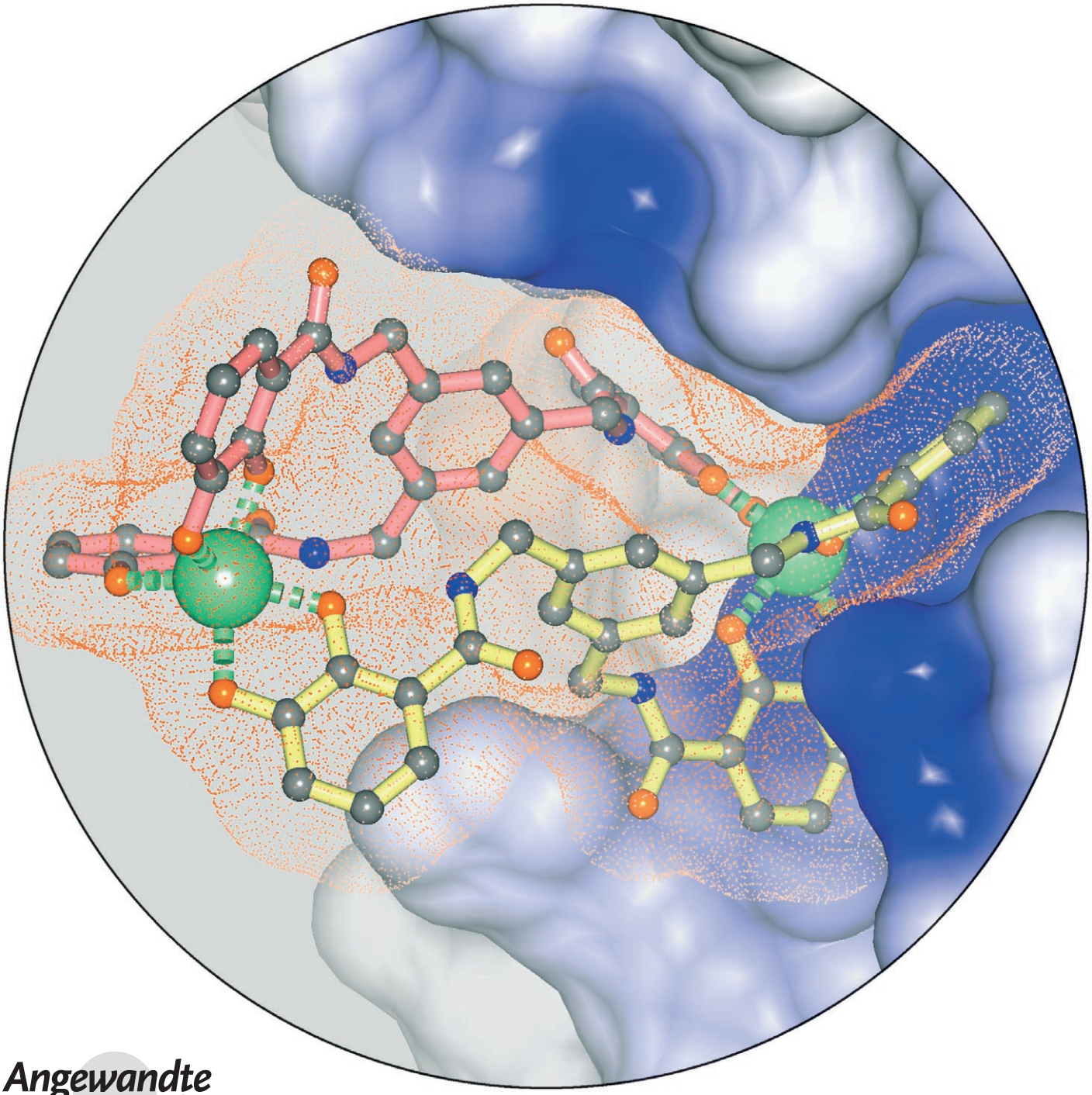
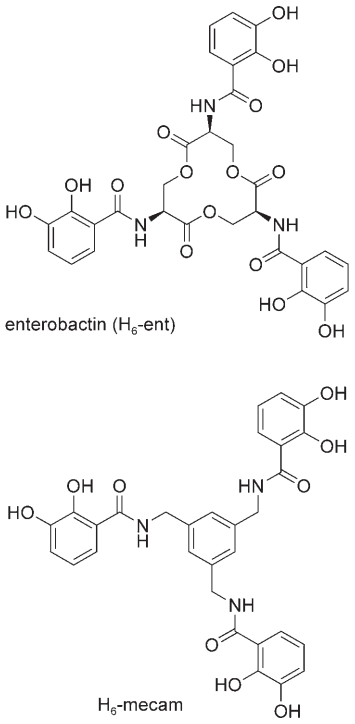


The control of self-assembly processes through noncovalent interactions is of immense importance in the area of bio-inspired supramolecular chemistry.[1] In particular, the exploration of interstrand π interactions to determine the nuclearity of helical metal complexes is currently attracting a lot of attention.[2,3] Here we present the crystal structure of the periplasmic ferric enterobactin binding protein CeuE of *Campylobacter jejuni* bound to Λ,Λ-[{Fe(mecam)}$_2$]$^{6-}$ (H_6-mecam = 1,3,5-*N*,*N*′,*N*″-tris(2,3-dihydroxybenzoyl)triaminomethylbenzene), which represents an intriguing example of a dinuclear ferric enterobactin mimic that is recognized stereoselectively by two identical protein molecules. The assembly is stabilized through hydrophobic interactions between the mesitylene spacers in the ligand backbone of the dinuclear iron complex.

C. jejuni is a leading cause of acute bacterial gastroenteritis. It is commensal in poultry, cattle, and swine and is normally transmitted to humans via contaminated poultry products or drinking water. To colonize the intestine of the host successfully, *C. jejuni* has to compete with other bacteria for essential nutrients, such as Fe.[4] To acquire Fe, *C. jejuni* uses a high-affinity uptake system, which relies on siderophores.[5] To maximize its chances of survival, *C. jejuni* takes advantage of several external siderophores adventitiously, including enterobactin (Scheme 1), the highly effective tris(catecholamide) siderophore of *Escherichia coli*.[6]

Enterobactin binds Fe^{III} with extremely high affinity ($\log\beta = 49$).[7] In Gram-negative bacteria, the Fe–enterobactin complex is recognized by a cell surface receptor protein[8] and transported into the periplasm, where it is captured by a periplasmic binding protein, CeuE in *C. jejuni*. The periplasmic ferric siderophore binding protein delivers the Fe–enterobactin complex to the inner membrane transporter for translocation into the cytoplasm.

As one of the most powerful iron chelators known, enterobactin has inspired the development of a range of synthetic tris(catecholamide) ligands, such as H_6-mecam[9] (Scheme 1). Like enterobactin, H_6-mecam relies on three catecholamide "arms" to chelate Fe^{III}, but the mesitylene backbone of H_6-mecam is hydrophobic and achiral while the tris(L-serine) backbone of enterobactin is hydrophilic and chiral. As H_6-mecam is easier to synthesize and more stable than the hydrolytically labile triester enterobactin, it has been used extensively to study various aspects of bacterial iron transport.[10,11] Ferric enterobactin and ferric mecam complexes, however, have so far eluded detailed structural characterization owing to their paramagnetism and the lack of good-quality single crystals. Crystal structures of Fe-tris(catecholamide) complexes are surprisingly rare, and a search of the Cambridge Structural Database revealed only one example, [Fe(trencam)]$^{3-}$,[12] in which the three Fe-binding catecholamides are attached to a triaminotriethylamine (tren) backbone.



Scheme 1. Enterobactin and its mimic, H_6-mecam.

Here, we determined the crystal structure of the periplasmic ferric enterobactin binding protein CeuE from *C. jejuni* in complex with ferric mecam. This structure is the first of a ferric mecam complex and the first to reveal the interactions of a periplasmic catecholamide siderophore receptor protein with its substrate (Figure 1). Remarkably, the siderophore mimic forms an [{Fe(mecam)}$_2$]$^{6-}$ dimer, which bridges a pair of CeuE molecules. This is a genuine bridge, as there are no protein–protein interactions in the (CeuE)$_2$-{Fe(mecam)}$_2$ assembly.

In the assembly, the two CeuE molecules are nearly identical and have bilobate structures characteristic of periplasmic binding proteins.[13] Each [Fe(catecholate)$_3$]$^{3-}$ recognition site of the central [{Fe(mecam)}$_2$]$^{6-}$ complex resides in a binding cleft between two α/β domains. The catecholate oxygen atoms interact with the positively charged sidechains of Arg117, Arg204, and Arg248, which balance the charge of the [Fe(catecholate)$_3$]$^{3-}$ unit (Figure 2). Although not apparent from the sequence, the overall structure of CeuE is very similar to that of FhuD, the periplasmic hydroxamate siderophore binding protein of *E. coli*.[14] In contrast to FhuD, the domain interface in CeuE is hydrophillic and selective for ferric catecholates, which are negatively charged.

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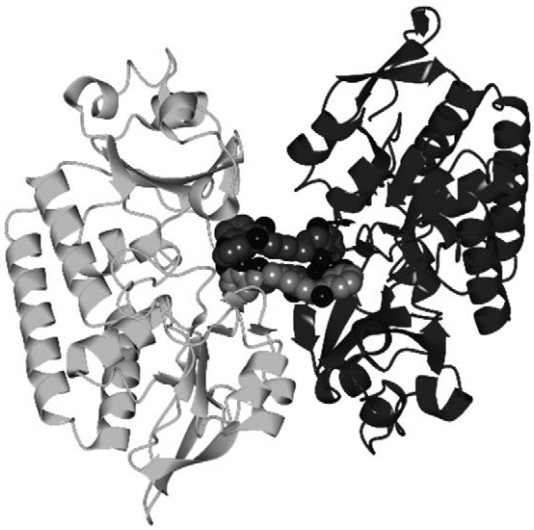

Figure 1. Ribbon representation of two CeuE molecules bound to the $[\{Fe(mecam)\}_2]^{6-}$ dimer, which is highlighted in space-fill representation with the carbon atoms of the two $mecam^{6-}$ ligands distinguished by shading.

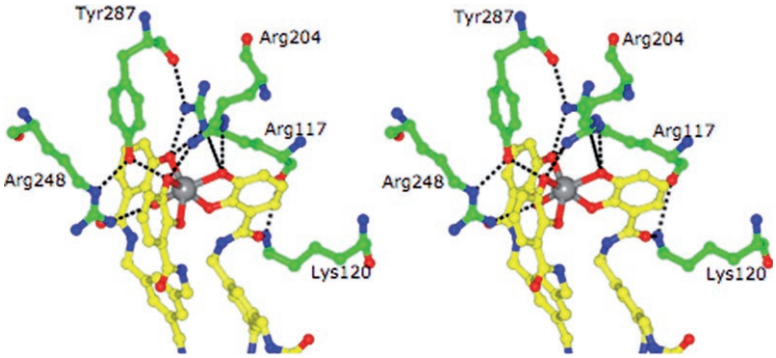

Figure 2. Stereoview showing the interactions of the binding pocket of CeuE (C green) with the $[Fe(catecholate)_3]^{3-}$ unit (C yellow, Fe gray).

Each iron atom in $[\{Fe(mecam)\}_2]^{6-}$ is coordinated by four oxygen donors from one mecam moiety and two from the other to result in a distorted octahedral coordination geometry (Figure 3). The Fe–O distances range from 1.9 to 2.2 Å. If we consider the resolution of the structure (2.4 Å), then these distances are in agreement with the distances found in $[Fe(trencam)]^{3-}$ (1.99 and 2.03 Å).[12] In the dimer, the aromatic rings of the two mecam backbones are nearly coplanar with an interplanar angle of 15°. The rings are significantly offset with one of the δ^+ methylene carbon atoms positioned over the center of the π electrons of the other ring—a geometry commonly observed in π-stacked systems.[15] The distance of 3.9 Å between the center of the aromatic ring and the methylene carbon atom indicates significant hydrophobic interactions.[15] Consequently, dimer formation appears to be favored by the hydrophobic nature of the mesitylene spacer of mecam, a structural feature of the mimic that is not present in enterobactin.

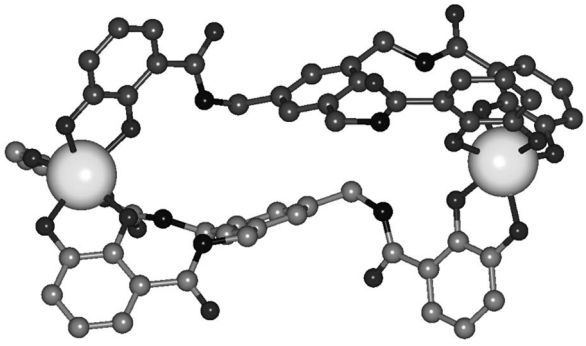

Figure 3. Ball-and-stick representation of the $[\{Fe(mecam)\}_2]^{6-}$ complex. Carbon atoms of the two $mecam^{6-}$ ligands are distinguished by shading, while hydrogen atoms are not shown.

In enterobactin, the conformation of the tris(serine) backbone would favor the hexadentate encapsulation of a single metal ion, a phenomenon that has been termed ligand predisposition.[16] Evidence for the mononuclear structure of $[Fe(ent)]^{3-}$ was obtained in vitro from NMR spectroscopic studies on the corresponding diamagnetic Ga^{III} complex,[17] crystallographic insights from the V^{IV} complex,[18] and MM3 calculations.[19]

In contrast to the tris(serine) and tren backbones, the aromatic spacer in mecam is planar and should allow the ligand to bind Fe^{III} in the form of a 1:1 or 2:2 complex or to form even larger aggregates. The immediate coordination environment of the Fe center is formally identical in these species. Information on the nuclearity of the Fe^{III} or Ga^{III} complexes formed by mecam in the absence of protein is not available in the literature. ^2H NMR spectroscopic studies on a mecam analogue with CD_3 groups in the 4-position of the catechol rings showed that upon Fe^{III} binding, only one paramagnetically shifted resonance was observed for the three CD_3 groups above pH 10,[20] indicating the formation of a C_3-symmetrical complex with equivalent catechol rings under these conditions. This result is consistent with the formation of a mononuclear complex, but also with a homochiral tetrahedral 4:4 complex[21b] or a mixture of different species in dynamic equilibrium on the NMR timescale.

In the presence of CeuE, dimerization to give $[\{Fe(mecam)\}_2]^{6-}$ is favored by hydrophobic interactions between the two spacer units which appear to outweigh the resulting sixfold negative charge of the metal complex, at least in forming the $(CeuE)_2$-$\{Fe(mecam)\}_2$ assembly, where the negative charge is countered by the protein, which minimizes the electrostatic repulsion in the dimer. A similar dependence of the nuclearity of metal complexes on spacer geometry and electrostatic effects is well documented for bis(catecholamide) complexes.[21] As these tetradentate ligands are unable to saturate the six coordination sites of Fe^{III} in a 1:1 complex, the formation of dinuclear triple-stranded $[Fe_2L_3]^{6-}$ helicates[22] or even tetrahedral $[Fe_4L_6]^{12-}$ clusters[21b] is observed. Which structure predominates depends on the type of spacer used.[21] If the geometrical constraints of the spacer are not unequivocal, mixtures of species are obtained and additional stabilizing effects, such as interactions with cationic guests,[23] π stacking,[2] solvent, and temperature,[24] determine the nuclearity.

Interestingly, in contrast to enterobactin, mecam delivers very little Fe to the cytoplasm. Instead, the Fe–mecam complex accumulates in the periplasmic space.[25] Assuming that Fe–mecam dimerizes in the presence of CeuE in the periplasm as it does in the crystals described here, the formation of dimeric assemblies could explain the lack of further transport. The concentration of about 0.5 mM of CeuE-Fe-mecam used in the crystallization procedure lies within the typical concentration range of substrate-bound binding proteins found in the periplasmic space.[13] Alternatively, the lack of further transport could be attributed to the protonation of one of the *meta*-hydroxy oxygen atoms in the Fe–mecam complex[20] ($\log K_{\mathrm{FeHmecam}} = 7.08$) in the slightly acidic periplasm, preventing transport across the inner membrane, as previously suggested.[25]

Another remarkable feature of the structure of the $(CeuE)_2$-$\{Fe(mecam)\}_2$ assembly is the chirality of the Fe centers. Both $[Fe(catecholate)_3]^{3-}$ recognition sites in the crystal structure of $[\{Fe(mecam)\}_2]^{6-}$ have the same chirality (Λ), resulting in a helical structure of the complex. This preference for the Λ configuration is retained in solution, as indicated by the circular dichroism (CD) spectrum in the region of the ligand-to-metal charge-transfer bands (Figure 4). A negative band around 450 nm and a positive band around 560 nm are characteristic of a tris(catecholamide) complex with Λ chirality.[26] As mecam is achiral and there are no direct contacts between the two CeuE molecules that could induce a helical twist in the complex, this preference results from interactions with the substrate-binding pocket of the protein.

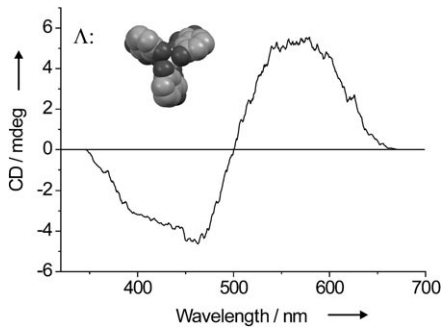

Figure 4. CD spectrum of the $(CeuE)_2$·$\{Fe(mecam)\}_2$ complex in buffer at pH 7.7. Inset: space-fill representation of the $[Fe(catecholate)_3]^{3-}$ unit illustrating the Λ configuration.

In contrast, the iron–enterobactin complex has been shown to adopt a Δ configuration induced by the chirality of the tris(L-serine) backbone. Correspondingly, the CD spectrum of the ferric enterobactin complex shows a positive band around 420 nm and a negative band at 530 nm.[26] According to reported experimental evidence,[17–19,26] ferric enterobactin is a mononuclear complex with Δ configuration in vitro, but it cannot be entirely excluded that ferric enterobactin may change configuration or even dimerize in the presence of a periplasmic binding protein. That ferric enterobactin can change configuration upon binding to a protein is evident from the crystal structure of the adduct between $[Fe(ent)]^{3-}$ and the lipocalin protein NGAL, in which the conformation of the Fe–tris(catecholamide) unit is Λ (PDB code 1L6M). It should be mentioned though that in the structure the chiral tris(L-serine) backbone was partially degraded.[27]

In conclusion, we have determined the crystal structure of a dimeric $(CeuE)_2$-$\{Fe(mecam)\}_2$ assembly that reveals interactions between the periplasmic binding protein CeuE of *C. jejuni* and a ferric enterobactin mimic, $[\{Fe(mecam)\}_2]^{6-}$. The protein induces Λ chirality at the Fe centers and balances the negative charge of the complex. The mesitylene spacer in the backbone of mecam, which differentiates the mimic from enterobactin, gives rise to hydrophobic interactions that favor the formation of the dimeric assembly. These results emphasize the importance of the backbone structure and chirality in the design of siderophore mimics.

Experimental Section

H_6-mecam was synthesized according to a reported procedure.[9b] M.p.: 129–133 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 4.46$ (d, 6H, $J = 6$ Hz, CH_2-NH), 6.66 (t, 3H, $J = 8$ Hz, ArH-cat), 6.75 (dd, 3H, $J = 1$ Hz, 8 Hz, ArH-cat), 7.19 (s, 3H, ArH-mes), 7.29 (dd, 3H, $J =$ 1 Hz, 8 Hz, ArH-cat), 9.38 ppm (t, 3H, $J = 6$ Hz, NH); ESI-MS (MeOH): *m/z* (%): 572.1 (100) $[M-H]^-$; elemental analysis (%) calcd for $C_{30}H_{27}N_3O_9 \cdot 0.5\,H_2O$: C 61.85, H 4.84, N 7.21; found: C 61.48, H 4.86, N 7.14.

The Fe^{III} complex of mecam was prepared by adding a solution of H_6-mecam (23 mg, 0.04 mmol) in methanol (1.25 mL) to a solution of $FeCl_3 \cdot 6\,H_2O$ (11 mg, 0.04 mmol) in H_2O (250 μL). The resulting dark purple solution was evaporated to dryness to afford a purple powder. ESI-MS (MeOH, apparent pH 3): *m/z* (%): 625.1 (100) $[M+2H]^-$, 647.1 (60) $[M+H+Na]^-$. HR ESI-MS: *m/z* calcd for $C_{30}H_{22}N_3O_9FeNa$: 647.0609; found: 647.0615. The absorption spectrum at pH 8 matched the one previously reported.[9b]

The crystal structure of $[\{Fe(mecam)\}_2]^{6-}$·$(CeuE)_2$ was refined against an X-ray data set, which is 92 % complete to 2.4-Å spacing and consists of 23425 unique reflections. The model has a working *R* factor of 0.17, a free *R* factor of 0.25, $\mathrm{rms}_{\mathrm{bond}} = 0.019$, and $\mathrm{rms}_{\mathrm{angle}} = 1.6°$. There are no Ramachandran outliers. Figures were generated with CCP4mg.[28] The atomic coordinates and X-ray crystal structure factor data have been deposited in the Protein Data Bank with the accession code 2chu. Details of protein expression, X-ray data collection, and refinement can be found in this deposition.

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