

Protein Crystallography through Supramolecular Interactions between a Lanthanide Complex and Arginine**

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The determination of the complete sequence of the human genome triggered the emergence of structural proteomics, a challenging field of research devoted to the determination of the structure of proteins at atomic resolution to understand their structure–function relationships. Among the various techniques for structure determination,^[1] X-ray crystallography plays an essential role. Its bottleneck is the serendipitous and time-consuming preparation of well-diffracting crystals. Once such crystals are obtained, de novo structure determination requires the preparation of at least one heavy-atom derivative. Nowadays, methods based on anomalous diffraction are routinely used.^[2] Lanthanide ions are thus very convenient for this purpose because of their large anomalous signal with either synchrotron or Cu_{Kα} radiation. Lanthanide derivative crystals of the protein may be prepared by: 1) replacing Ca²⁺ by Ln³⁺ ions in calcium-binding proteins,^[3] 2) covalent grafting of dysprosium complexes^[4] or a terbium binding tag,^[5] or 3) cocrystallization with macrocyclic gadolinium, ytterbium, or lutetium complexes.^[6] The last method is very easy to carry out, but the interactions between the lanthanide complexes and the protein are difficult to predict. To shed light on this problem, we recently developed a research program devoted to the study of the solid-state and solution interactions between lanthanide complexes and biological macromolecules.

Herein we describe the properties of tris(dipicolinate)-lanthanide complexes [Ln(dpa)₃]^{3−} (dpa = dipicolinate = pyridine-2,6-dicarboxylate),^[7] which could be highly interesting for macromolecular crystallography, since 1) successful derivatization can easily be detected through the intrinsic luminescence of the europium and terbium complexes, 2) the new crystal form of derivatives with hen egg-white lysozyme (HEWL) evidenced strong interactions between the tris(dipicolinate)lanthanide complexes and the protein; these interactions were shown to involve guanidinium moieties of arginine residues, and 3) the occurrence of these supramolecular interactions occurred both in aqueous solution as well as in the solid state, with the ethylguanidinium cation (EtGua⁺) mimicking the behavior of accessible arginine groups of proteins.

Cocrystallized derivative crystals of HEWL were obtained by the hanging-drop method. Mixing HEWL and A₃[Ln(dpa)₃] (Figure 1 a, Ln = Eu, Tb; A = Li, Na, Cs, NH₄) led to a precipitate from which crystals grew within a few days. The shape of the resulting crystals (Figure 1 b) was very different from that of native tetragonal crystals obtained under similar conditions.^[8] Irradiation with UV light (λ_{ex} =

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

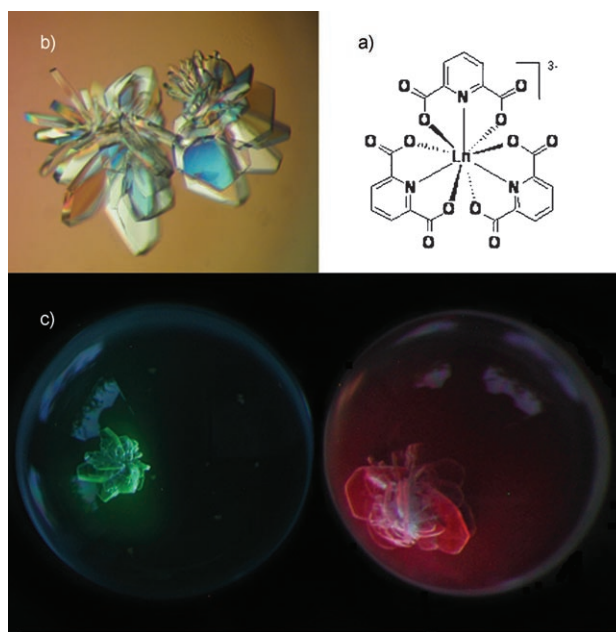


Figure 1. a) Chemical structure of the [Ln(dpa)₃]^{3−} complex, b,c) microscopy views of derivative crystals of HEWL obtained with Na₃[Ln(dpa)₃]: b) under polarized visible light (×40) and c) under irradiation with UV light (×25) in complex-free drops: Ln = Tb (green crystals, left view), Ln = Eu (red crystals, right view).

254 nm) promotes the green and red luminescence of terbium and europium, respectively (Figure 1 c),^[7b] which showed that successful derivatization had been achieved.

The derivative crystals belong to monoclinic space group *C*2 ($a = 50.27$, $b = 33.78$, $c = 69.68$ Å, $\beta = 108.22^\circ$). The crystal structure of the Eu derivative was solved by using the single-wavelength anomalous diffraction (SAD) method on data collected in-house to a resolution of 1.5 Å. The crystal packing is shown in Figure 2. Data processing and structure-

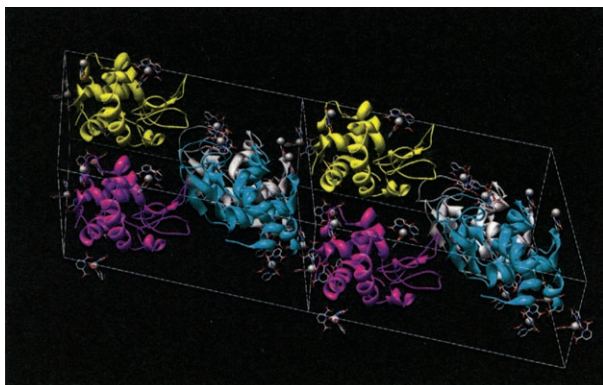


Figure 2. Monoclinic crystal packing in the Eu derivative crystal of HEWL.

refinement statistics are given in the Supporting Information. Five $[\text{Eu}(\text{dpa})_3]^{3-}$ sites were found per HEWL monomer, with binding sites located at the interface between protein molecules. The three major sites are shown in Figure 3. The highest occupancy values (sites 1 and 2) are close to 1. Site 2 is located on a crystallographic twofold axis. The fixation of $[\text{Eu}(\text{dpa})_3]^{3-}$ complexes on HEWL is enantioselective for the four most occupied sites in which all the dpa ligands were modeled. Only one dpa ligand was modeled in the weak electron density of the least occupied site. $[\text{Eu}(\text{dpa})_3]^{3-}$ complexes link the protein through hydrogen bonds between the oxygen atoms of the carboxylate groups of dpa and the protein hydrogen-bond donors such as amido, amino, and hydroxy groups, as shown in Figure 3. Water molecules complete the hydrogen-bonding network of the dpa groups. Interestingly, the three major sites are surrounded by at least two arginine residues (Figure 3). Moreover, eight of the eleven arginine residues of the HEWL are hydrogen bonded to dpa, thus suggesting their involvement in specific interactions. The high affinity of the complex for arginine residues can be explained by: 1) electrostatic interactions between tris(anionic) complexes and cationic arginine residues and 2) a well-defined hydrogen-bonding network in which guanidinium moieties act either as monodentate or as bidentate hydrogen-bond donors (Figure 3). HEWL is known to display many crystal forms, depending on the crystallization conditions (pH, temperature, additive agents). Since arginine residues are frequently involved in protein–protein interfaces,^[9] polymorphism of HEWL was suggested to be related to its high arginine content (11 of 129 residues).^[10] Furthermore, anions in solution affect both the solubility of proteins and the crystal packing by interacting with positively charged amino acids.^[11] In the derivative crystal, the complexes are located at

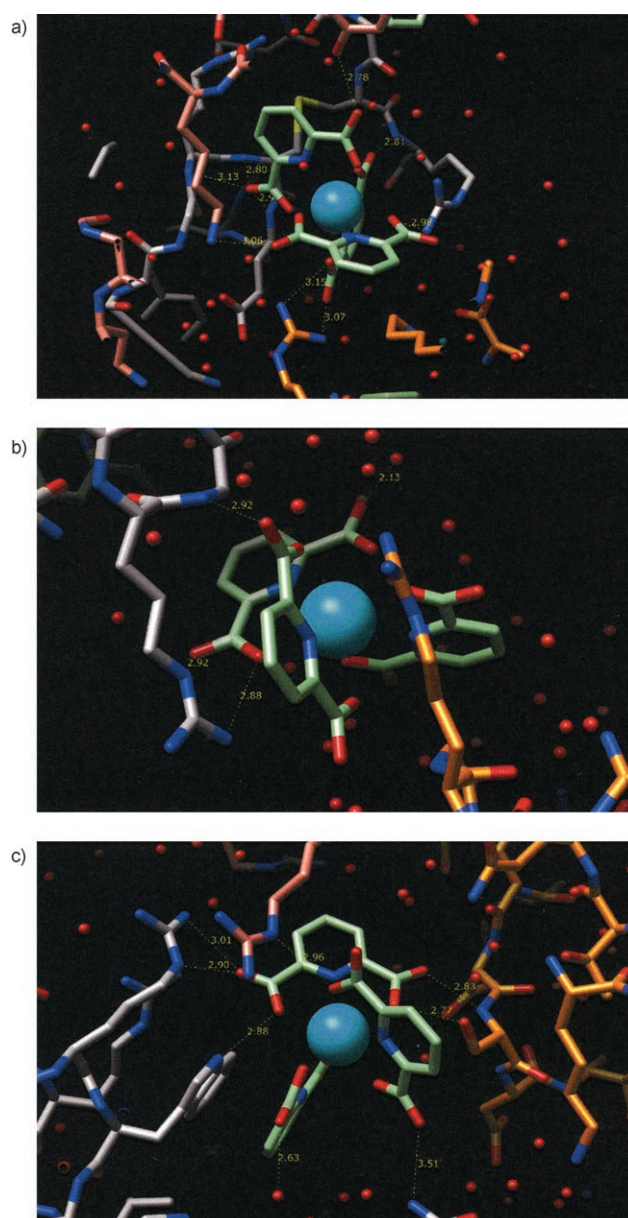


Figure 3. Main binding sites of $[\text{Eu}(\text{dpa})_3]^{3-}$ in the crystal of the HEWL derivative. The Eu atoms are shown in blue, dpa ligands in light-green, and symmetrical HEWL molecules in different colors. A) Site 1: molecule A (x, y, z) in white, molecule B ($x+1/2, y-1/2, z$) in salmon, molecule C ($x, y-1, z$) in orange; b) Site 2: molecule A (x, y, z) in white, molecule D ($1-x, y, -z$) in orange; c) Site 3: molecule A (x, y, z) in white, molecule E ($x, y+1, z$) in orange, molecule F ($-x+1/2, y+1/2, -z+1$) in salmon.

the interface between protein molecules. Therefore, the new crystal packing is due to original intermolecular contacts between the lanthanide complex and hydrogen-bond donor groups, especially arginine residues.

In view of the location of the $[\text{Eu}(\text{dpa})_3]^{3-}$ complexes in the HEWL derivative crystals, which provide evidence for the affinity of the complex for arginine residues, we performed experiments that showed supramolecular association between the complex and the EtGua^+ ions, which mimic the arginine side chain.

The reaction between $\text{Cs}_3[\text{Tb}(\text{dpa})_3] \cdot 7\text{H}_2\text{O}$ and ethylguanidine hydrochloride (EtGuaCl) in water produced transparent crystals of $(\text{EtGua})_3[\text{Tb}(\text{dpa})_3] \cdot 2\text{H}_2\text{O}$. The crystal data and refinement parameters are summarized in the Supporting Information. The predominant interaction modes are depicted in Figure 4. The EtGua^+ ion acts as a bidentate

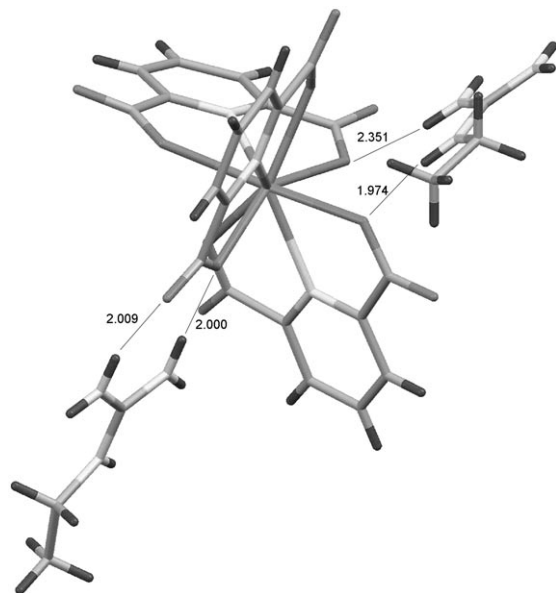
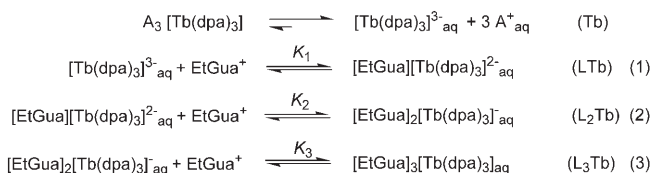


Figure 4. Selected interactions between $[\text{Tb}(\text{dpa})_3]^{3-}$ and EtGua^+ ions (H bonds are indicated by dashed lines).

hydrogen-bond donor, and forms an eight-membered ring with a Tb–carboxylate fragment ($\text{N} \cdots \text{H} \cdots \text{O}$ distances: ca. 2 Å). The crystal structure reveals that the EtGua^+ ions mimic the interactions observed in derivative crystal of HEWL, where arginine residues interact with the complex through longer mono- or bidentate hydrogen bonds (in the range 2.5–3 Å; Figure 3). The differences in distances and the large number of water molecules remaining in the second coordination sphere of the complex in the derivative crystal of HEWL can be easily explained by the large steric hindrance of the protein molecule compared to that of the EtGua^+ ions. Finally, the strong interactions between the $[\text{Ln}(\text{dpa})_3]^{3-}$ and guanidinium ions can explain why the complex acts as a keystone in the crystal packing of the protein derivative by strongly connecting different macromolecules together, thereby forming a new crystal lattice.

Furthermore, the EtGua^+ model described above allows analysis of interactions in the derivative crystal of HEWL not only in the crystalline state but also in aqueous solution. We thus employed terbium complexes as paramagnetic NMR shift reagents to undertake ^1H NMR chemical shift titration experiments. Indeed, the strong pseudocontact shifts induced by terbium ions, which can be detected as far as 40 Å away from the metal center, are particularly well suited to reveal long-range interactions such as those occurring in the second coordination sphere of complexes (see above).^[12,13] The addition of EtGuaCl to a solution of $\text{A}_3[\text{Tb}(\text{dpa})_3] \cdot x\text{H}_2\text{O}$ ($\text{A} = \text{Na}, \text{Li}, \text{Cs}$, $6 < \text{pH} < 7$) in deuterated water at room

temperature resulted in significant up-field shifts of the NMR signals from the protons of the ethyl chain. The magnitudes of these shifts decrease with increasing amount of EtGua^+ ions, and in all cases only one set of signals is observed for each set of protons (see Figure S1 in the Supporting Information).^[14] These observations indicate that the EtGua^+ ion is actually in rapid equilibrium in water between its solvated state and chemical species resulting from its interaction with the terbium complex. In addition, for high n_{eq} ratios ($n_{\text{eq}} = [\text{EtGuaCl}]/[\text{Cs}_3[\text{Tb}(\text{dpa})_3]]$), all coordination sites on the $[\text{Tb}(\text{dpa})_3]^{3-}$ ion are progressively occupied, and thus “free” EtGua^+ ions are accumulated in the solution. Similar studies involving lanthanide ions have already been performed in the past using ^1H (or ^{13}C) chemical shift titration experiments, notably for 1:1 and 2:1 complexes.^[15] In the present case, the system is best modeled by a series of three equilibria to form the 1:1, 2:1, and 3:1 $\text{EtGua}^+ / [\text{Tb}(\text{dpa})_3]^{3-}$ adducts (LTb , L_2Tb , and L_3Tb with rate constants K_1 , K_2 , and K_3 , respectively; Scheme 1).^[16] Similar to other studies, a nonlinear fit of the



Scheme 1. Schematic representation of the interaction between $\text{A}_3[\text{Tb}(\text{dpa})_3]$ ($\text{A} = \text{Li}, \text{Na}, \text{Cs}$) and EtGua^+ ions in water, which has been modeled according to the ^1H NMR data. For clarity, water molecules that are involved in the solvent sphere of the terbium complex have been omitted.

shift deviation over a “coarse grid search” of the parameter space leads to the determination of the whole set of thermodynamic constants and NMR parameters within an accuracy that mainly depends on the experimental uncertainty of the shift measurements.^[17]

In this model, all the ion pairs are presumed to be fully solvated in aqueous solution, and the contributions of counterions to the equilibrium constants are neglected. Each proton i ($i = 1, 2$) undergoes a shift of ∂_i^{free} when the EtGua^+ counterion is in solution, and a shift of ∂_i^{comp} when it belongs to the second coordination sphere of the terbium complex with a 1:1, 2:1, or 3:1 ratio. Under these conditions, the experimental ^1H NMR chemical shift ∂_i^{exp} ($i = 1, 2$) can be expressed as Equation (4):

$$\partial_i^{\text{exp}} = \frac{[\text{L}]}{n_{\text{eq}} C_0} \partial_i^{\text{free}} + \frac{\{[\text{LTb}] + 2[\text{L}_2\text{Tb}] + 3[\text{L}_3\text{Tb}]\}}{n_{\text{eq}} C_0} \partial_i^{\text{comp}} \quad (4)$$

where $[\text{L}]$ and $[\text{L}_j\text{Tb}]$ ($j = 1, 2, 3$) represent the concentrations of free EtGua^+ ions and EtGua^+ associated with the complex, respectively. The ∂_i^{free} ($i = 1, 2$) chemical shifts were determined as described in the experimental section, and the best fit of the experimentally measured proton shifts upon titration allowed the determination of the three thermodynamic constants K_1 , K_2 , and K_3 as well as ∂_i^{comp} ($i = 1, 2$). To estimate the overall uncertainty that results from our data-

analysis method and from the experimental errors, we used a series of eight titration curves (Figure 5 and Supporting Information) with different counterions at different concentrations C_0 as a statistical ensemble to determine the following average values (and their corresponding standard deviations):

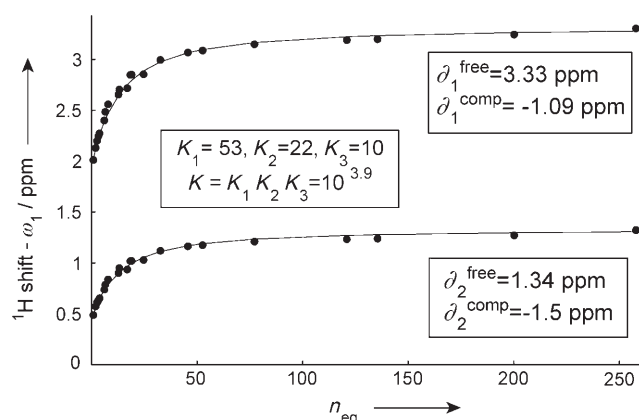


Figure 5. Variation in the experimental proton shifts versus n_{eq} , and a model curve that best fits the experimental data. $C_0 = 9.1 \times 10^{-3} \text{ mol L}^{-1}$.

$\delta_1^{\text{comp}} = -0.6$ (0.5) ppm, $\delta_2^{\text{comp}} = -1.15$ (0.45) ppm, $\delta_1^{\text{free}} = 3.28$ (0.04) ppm, and $\delta_2^{\text{free}} = 1.30$ (0.04) ppm. The value of the average overall complexation constant, $K = K_1 K_2 K_3 \approx 10^{3.7(0.2)}$ [$K_1 = 61$ (11), $K_2 = 17$ (4), $K_3 = 4$ (0.5)] clearly indicates a strong interaction between the EtGua⁺ ions and the Tb complex in aqueous solution.

In conclusion, the high value of the association constant between $[\text{Ln}(\text{dpa})_3]^{3-}$ and EtGua⁺ ions results from a strong interaction between the complex and the guanidinium moieties through a hydrogen-bonding network, and also evidenced in both the HEWL and EtGua⁺ crystals. This high affinity can account for the novel crystal packing of the derivative crystal of HEWL, in which lanthanide complexes are located at the interface between protein molecules. This supramolecular interaction, also observed in crystals of thaumatin derivative,^[18] could be ubiquitous and therefore should be interesting for the preparation of derivative crystals of protein with high phasing power, in which derivatization could be verified by simple observation through the use of the luminescence properties of the complex.

Experimental Section

Protein crystallography: HEWL was purchased from Boehringer and used without further purification. Derivative crystals were obtained by using the hanging-drop method with HEWL (30 mg mL⁻¹), Na₃[Ln(dpa)₃] (50–100 mM) in 100 mM β-2-[N-morpholino]ethanesulfonic acid (MES) buffer (pH 5.1) and NaCl (200–300 mM). The

structure of the derivative has been deposited in the Protein Data Bank (ID 2PC2).

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