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Binding and Stabilization of a Semiquinone Radical by an Artificial Metalloenzyme Containing a Binuclear Copper (II) Cofactor

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A binuclear Cu(II) cofactor was covalently bound to a lauric acid anchor. The resulting conjugate was characterized then combined with beta-lactoglobulin (βLG) to generate a new biohybrid following the so-called “Trojan horse” strategy. This biohybrid was examined for its effectiveness in the oxidation of a catechol derivative to the corresponding quinone. The resulting biohybrid did not exhibit the sought after catecholase activity, likely due to its ability to bind and stabilize the

semiquinone radical intermediate DTB-SQ. This semi-quinone radical was stabilized only in the presence of the protein and was characterized using optical and magnetic spectroscopic techniques, demonstrating stability for over 16 hours. Molecular docking studies revealed that this stabilization could occur owing to interactions of the semi-quinone with hydrophobic amino acid residues of βLG.

Introduction

During the last decades, new hybrid biocatalysts, which combine the advantages of biocatalysis and homogeneous catalysis, have been developed, namely artificial metalloenzymes (AMEs).^[1] These associate a synthetic metal-cofactor responsible for a catalytic activity with a protein that provides a chiral environment and helps prevent the degradation of the cofactor. Like enzymes, AMEs can operate under mild conditions, and like synthetic catalysts, they can be used for the catalysis of a wide range of reactions.^[1] In addition, the efficiency and the selectivity of an AME can be optimized either

by directed evolution,^[2] or by redesign of metal cofactors via chemical modifications. Different strategies have been described to obtain AMEs. Firstly, the direct insertion of a metal ion into proteins has led to systems that proved to be able to catalyze various reactions such as the epoxidation and the hydroformylation of alkenes,^[2,3] the reduction of superoxide,^[4] as well as oxo-transfer reaction,^[5] and peroxidase-like reactions.^[6] Secondly, the covalent attachment of a metal cofactor into a protein, most often by a covalent bond with a cysteine residue, provided AMEs that have been used to catalyze a wide range of enantioselective transformations, such as ester hydrolyses,^[7] sulfoxidations,^[8] hydrogenations,^[9] Diels-Alder cycloadditions,^[10] olefin metathesis,^[11] and cyclopropanations.^[12] A third strategy, named “Trojan horse” strategy, is based on the strong affinity of a protein for a molecule (ligand, inhibitor, etc.) that is bound to the metal complexes for vectorization towards the binding site. This strategy was historically introduced by Whitesides and co-workers^[13] and was mainly developed and optimized by Ward and collaborators, taking advantage of the strong affinity of (strept)avidin for biotin ($K_D = 10^{-14}$ M). The latter was used as a “Trojan Horse” to anchor non-covalently numerous metal complexes inside (strept)avidin binding site thus providing access to new enzymatic activities.^[14] Since the 2000s, this strategy has been regularly used to build different AMEs using different ligand/protein couples such as: antigen/antibody,^[15] testosterone/neocarzinostatin NCS-3.24 variant,^[16] ibuprofen/human serum albumin (HSA),^[17] and more recently, fatty acid/beta-lactoglobulin (βLG).^[18]

In the last example, Salmain *et al.* exploited the affinity of βLG for fatty acids to design artificial metalloenzymes. βLG, a small protein found in cow's milk, belongs to the lipocalin protein family as it exhibits a typical lipocalin fold with an eight-stranded (labeled from A to H in Figure 7a) β-barrel domain comprising a calyx-shaped cavity. βLG is able to

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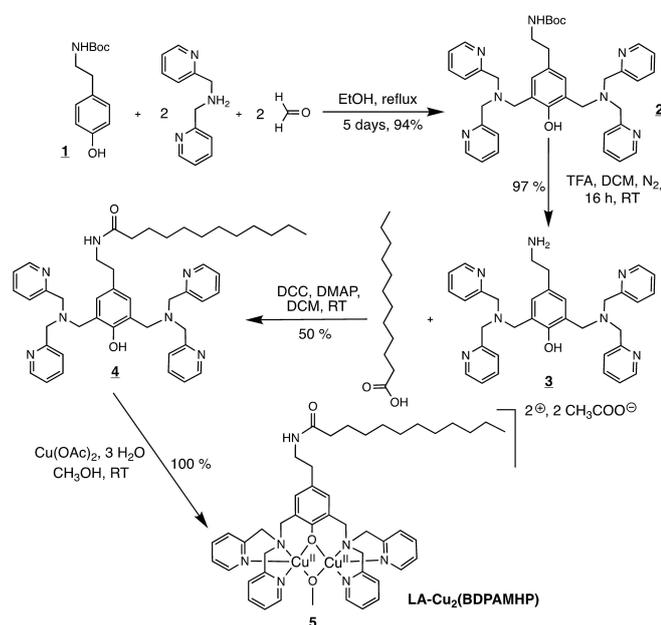
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transport hydrophobic ligands such as retinoids and fatty acids with sub-micromolar dissociation constants and a stoichiometry close to 1:1. fatty acids of varying sizes get accommodated in β LG with the carbon chain laying inside the cavity and the carboxylic acid function oriented towards its entrance.^[19] The access to this cavity is regulated by the Tanford transition i.e. a conformational change of an EF-hand at a pH of about 7.0.^[20] Taken altogether, these properties made β LG a very good candidate for the construction of AMEs using the Trojan Horse strategy. Indeed, Michelle Salmain's team designed Trojan horses by binding the carboxylic acid function of fatty acids to an amine substituent of a 2,2'-dipyridylamine (DPA)-type Ru or Rh complex. The catalytic activity of the biohybrid, which was constructed by incorporating the fatty acid-DPA-Ru or -Rh complex into β LG, was tested for the asymmetric hydrogenation reaction of an aryl ketone. The different biohybrids were found capable of carrying out the transfer hydrogenation of trifluoroacetophenone (TFAP) with up to 100% yield and up to 32% enantiomeric excess.^[18]

Based on these observations, we decided to use the Trojan horse strategy to build new artificial metalloenzymes based on β LG that enlarge the scope of catalysis to other reactions such as oxidation reactions that are compatible with catalysis by metalloproteins in aqueous media. Particularly, we associated β LG with a binuclear copper complex $\text{Cu}_2(\text{BDPAMHP})$ functionalized with a lauric acid (LA) tail (Scheme 1) thus forming a new



Scheme 1. Synthesis of di-copper(II)-N-(3,5-bis((2,2'-dipicolylaminomethyl)-4-hydroxy-phenethyl)lauramide LA- $\text{Cu}_2(\text{BDPAMHP})$ **5**.

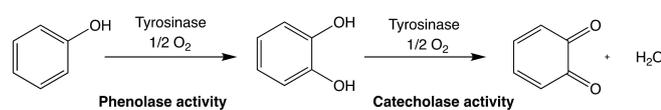


Figure 1. Phenolase and catecholase activities of tyrosinases.

Trojan horse with the aim to get an AME that would present tyrosinase-like activity.

Tyrosinases (Ty, EC 1.14.18.1) are copper-containing metalloenzymes that are widely distributed in microorganisms, plants and animals, wherein they catalyze the oxidation of phenolic compounds to catechols (phenolase activity) and of catechols to *o*-quinones (catecholase activity) (Figure 1).^[21]

Many biomimetic dinuclear copper(II) complexes that show a good catecholase activity have been reported in the literature without however constituting good mimics of the metalloprotein. Indeed, they lack the interactions with the second coordination sphere amino acids that can allow, in metalloproteins, the stabilization of highly reactive species that lead to an increase in their lifespan. On the contrary, it has recently been reported by the groups of DeGrado^[22] and Roelfes^[23] that semiquinone radicals were stabilized inside cavities of AMEs as intermediate metal-semiquinone complexes. Indeed, the 3,5-di-tert-butylsemiquinone (DTB-SQ) radical resulting from the one-electron oxidation of 3,5-di-tert-butylcatechol (DTBC) was stabilized inside a de novo engineered metalloprotein [DFsc-Zn^{II}]^[22] and inside artificial metalloproteins formed by the incorporation of 2,2'-bipyridylalanine Cu(II), Fe(II), Ni(II), and Zn(II) complexes into the lactococcal multidrug resistance regulator (LmrR).^[23]

The results reported in this manuscript reveal that the AME β LG-LA- $\text{Cu}_2(\text{BDPAMHP})$ did not show any catecholase activity. However, we attributed this peculiarity to its ability to stabilize the DTB-SQ radical intermediate, as demonstrated by UV-vis spectroscopy analysis as well as by EPR characterization of the formed DTB-SQ metal complex. This study serves as a starting point for further investigations aimed at better understanding the intricate mechanisms governing catalytic reactions involving the β LG-LA- $\text{Cu}_2(\text{BDPAMHP})$ AME.

Results and Discussion

Synthesis of the Di-Copper(II)-N-(3,5-Bis((2,2'-Dipicolylaminomethyl)-4-Hydroxy-Phenethyl)lauramide, LA- $\text{Cu}_2(\text{BDPAMHP})$ **5**

LA- $\text{Cu}_2(\text{BDPAMHP})$ **5** was prepared in four steps as described in Scheme 1.

First, the reaction of carbamate **1** with 2.8 equiv. of 2,2'-dipicolylamine (DPA) and 2.8 equiv. of formaldehyde under reflux for 6 days in 12 mL ethanol afforded, after purification by chromatography on a silica gel column, tert-butyl (3,5-bis((2,2'-dipicolylaminomethyl)-4-hydroxyphenethyl) carbamate **2** as a brown oil in a 94% yield. Further, the deprotection of the amine function by treatment of **2** by trifluoroacetic acid in dichloromethane (DCM) for 16 h under N_2 at room temperature led to 4-(2-aminoethyl)-3,5-bis((2,2'-dipicolylaminomethyl)phenol **3** as an oil in a 97% yield. To introduce the fatty acid chain, lauric acid was allowed to react with **3** in the presence of 1 equiv. of 1,1'-carbonyldiimidazole and 0.1 equiv. of DMAP in DCM at room temperature. After 18 h of reaction, N-(3,5-bis((2,2'-dipicolylaminomethyl)-4-hydroxyphen-ethyl)lauramide **4** was

obtained, after work-up and purification by chromatography on a silica gel column, as a yellow oil in a 50% yield. Finally, reaction of **4** with 2 equiv. of copper (II) acetate in methanol afforded the LA-Cu₂(BDPAMHP) complex **5** in a quantitative yield. All of the synthesized organic compounds **1–4**, were characterized by NMR spectroscopy and by HR ESI-MS, whereas the LA-Cu₂(BDPAMHP) complex **5** was characterized by HR ESI-MS.

Study of the Affinity of β LG for the LA-Cu₂(BDPAMHP) Complex **5** by Quenching of Intrinsic Fluorescence

The affinity of β LG for the LA-Cu₂(BDPAMHP) complex **5** was determined by the quenching of the fluorescence of its tryptophan residues caused by the interaction of this copper complex with β LG. The excitation wavelength was set at $\lambda_{\text{exc}} = 290$ nm, which corresponds to the wavelength at which photons are absorbed by the tryptophan residues, and the emitted fluorescence was analyzed at an emission wavelength $\lambda_{\text{em}} = 345$ nm.

The fluorescence of a 5 μ M solution of the betalactoglobuline in 20 mM HEPES buffer pH 7.5, was thus measured in the presence of increasing amounts of a 0.5 mM solution of LA-Cu₂(BDPAMHP) in acetonitrile, so that the final proportion of acetonitrile should not exceed 10%. As shown in Figure 2, an increase in the concentration of metal complex caused a decrease of the fluorescence of the protein at 345 nm.

The dissociation constant K_D of the β LG-LA-Cu₂(BDPAMHP) complex, was determined from the curve representing the decrease in fluorescence at 345 nm ($\Delta F = F_0 - F$) as a function of the total ligand concentration ($[\text{LA-Cu}_2(\text{BDPAMHP})]_{\text{tot}}$) in the solution (Figure 2, inset) as described in Materials and Methods. The continuous curve shown in Figure 2 corresponds to the optimal fit with a single site model resulting in $K_D = 0.76 \pm$

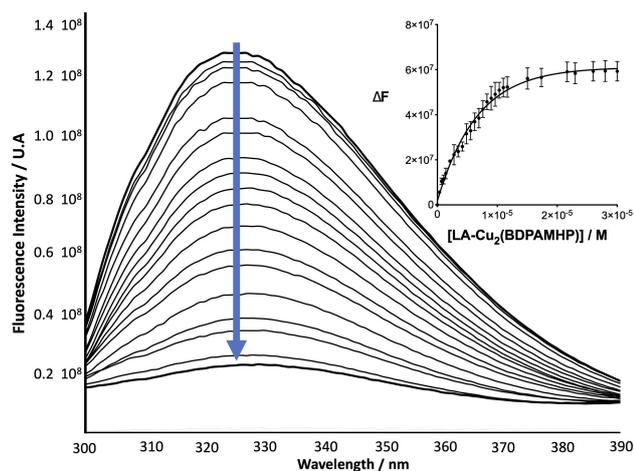


Figure 2. Fluorescence titration of LA-Cu₂(BDPAMHP) **5** binding to β LG: intrinsic tryptophane fluorescence emission spectra of β LG (5 μ M in 20 mM HEPES buffer pH 7.5, $\lambda_{\text{ex}} = 290$ nm), in the presence of increasing concentrations of **5** (0 to 0.3 mM, from top to bottom). Inset: corresponding emission signal at 345 nm fitted with equation 1 as described in Materials and Methods.

0.07 μ M, $n = 0.91 \pm 0.02$. This clearly showed that only one molecule of LA-Cu₂(BDPAMHP) was bound in the binding pocket of β LG with a good affinity. As shown in Table 1, this stoichiometry is close to those reported for the binding to β LG of lauric acid ($n = 0.86 \pm 0.05$) and of a rhodium complex bearing a lauric acid tail, LA-RhDPA(Cp)(Cl) ($n = 0.93 \pm 0.02$).^[18] In addition, since the corresponding K_D value (0.76 ± 0.07 μ M) is also close to those reported for these two complexes ($K_D = 0.7 \pm 0.16$ and $K_D = 0.66 \pm 0.05$ μ M resp.), it is clear that the attachment of the lauric acid chain to the copper complex does not alter its affinity for β LG.

The association of LA-Cu₂(BDPAMHP) with one equivalent of β LG led to the β LG-LA-Cu₂(BDPAMHP) biohybrid which was characterized by a UV-visible spectrum that displayed a weak band at 415 nm (ϵ ($\text{M}^{-1} \text{cm}^{-1}$) = 1400) (Figure S14).

Catecholase Activity

The catecholase activity of the β LG-LA-Cu₂(BDPAMHP) AME was assayed and compared to that of the cofactor alone, using 3,5-Di-Tert-ButylCatechol (DTBC) as a substrate (Figure 3).

Table 1. Apparent dissociation constants (K_D) of β LG-LA-metal-ligand complexes and apparent metal complex- β LG stoichiometry calculated from fluorescence titration measurements.

Complex	K_D (μ M)	n	Ref.
LA-RhDPA(Cp)(Cl) ^[a]	0.66 ± 0.05	0.93 ± 0.02	[18]
LA-Cu ₂ (BDPAMHP) ^[b]	0.76 ± 0.07	0.91 ± 0.02	This work
Lauric acid (LA)	0.7 ± 0.16	0.86 ± 0.05	[18]

[a] DPA = 2,2'-dipyridylamine, Cp = cyclopentadienyl. [b] BDPAMHP = N-(3,5-bis((2,2'-dipicolylaminomethyl)-4-hydroxyphenethyl).

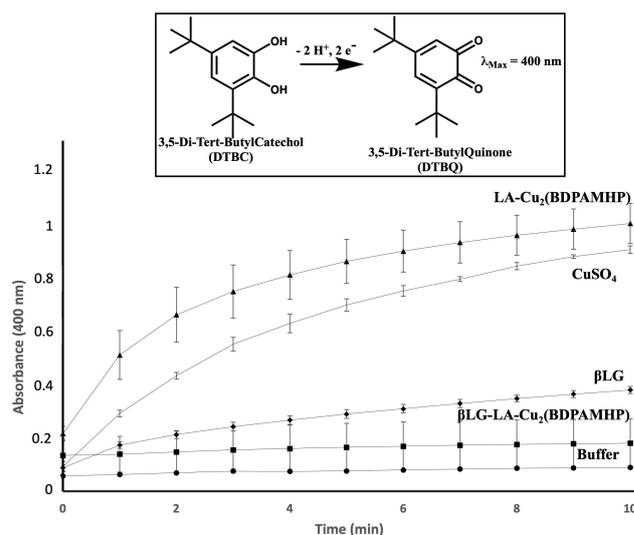


Figure 3. Catecholase activity of LA-Cu₂(BDPAMHP) **5** and of the β LG-LA-Cu₂(BDPAMHP) AME. 10 μ M solutions of LA-Cu₂(BDPAMHP) or of the AME in 0.1 M Tris-HCl buffer, pH 7.5 were treated at RT with 1 mM solutions of DTBC in MeCN and the evolution of the absorbance at 400 nm of formed DTBQ was recorded as a function of time.

As shown in Figure 3, both CuSO_4 and the copper complex $\text{LA-Cu}_2(\text{BDPAMHP})$ were able to catalyze the oxidation of DTBC to the corresponding quinone. However, when this complex was inserted into beta-lactoglobulin, almost no catecholase activity could be detected. This absence of catalysis could be explained by the stabilization of the semiquinone DTB-SQ radical anion reaction intermediate, which could prevent the reaction from proceeding further to yield the quinone.

Radical Stabilization

DeGrado and his colleagues,^[22] followed by G. Roelfes *et al.*^[23] discovered that artificial metalloenzymes could efficiently stabilize the DTB-SQ radical anion produced by oxidation of 3,5-di-tert-butylcatechol (DTBC). Subsequently Yoon *et al.* showed that the radical thus formed is stabilized by the metal ion, hydrophobic sequestration and interactions with hydrophilic residues inside the protein.^[25] Such a radical could be generated by reaction of equimolar amounts of the catechol DTBC and the quinone DTBQ through a disproportionation reaction. DTB-SQ was reported to present two absorption bands between 740–765 and 820–850 nm.^[22,23,25] and its formation can be followed by UV-visible spectroscopy. The formation of DTB-SQ radical was thus followed by UV-visible spectroscopy, and the evolution of its absorbance at 753 nm was monitored as a function of time, during the disproportionation of a 0.5 mM 1/1 DTBC/DTBQ mixture in the presence either of 10 μM $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$, of 10 μM $\beta\text{LG-LA}(\text{BDPAMHP})$ or of 10 μM βLG .

It appeared that in the presence of the $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$ the absorbance at 416 nm due to the starting DTQB substrate (Figure S17 of the SI) decrease gradually with time whereas the absorbance at 468, 753 and 826 nm due to the formation of DTB-SQ increased in the first 50 min of reaction with isobestic points at 362 and 487 nm (Figure 4) and then stabilized to remain stable for at least 18 h. This spectral evolution was similar to that reported by DeGrado and his colleagues,^[22] who observed a large decrease of the Q band ($\lambda_{\text{max}} = 415$ nm) and the appearance of a new broad band that spanned 740–850 nm when $[\text{DFsc-Zn}(\text{II})_2]$ was added to an equimolar mixture of Q and QH_2 , with an isobestic point at 492 nm that suggested the lack of additional intermediates.

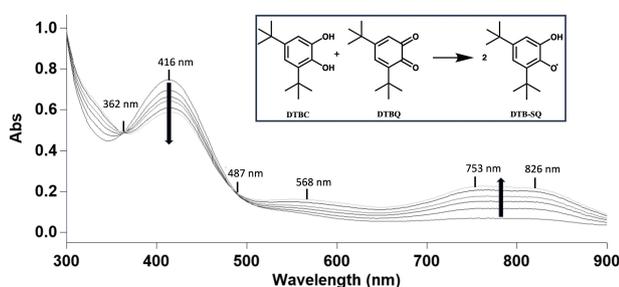


Figure 4. Evolution of the UV/visible spectrum of a 1/1 mixture of 0.5 mM DTBC and 0.5 mM DTBQ in the presence of 10 μM $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$ in 0.1 M Tris-HCl buffer, pH 7.5 containing 10% DMSO. Spectra were recorded during 18 hours at a frequency of 1 scan every 30 min.

On the contrary in the presence of either $\beta\text{LG-LA}(\text{BDPAMHP})$ or of βLG no noticeable evolution of the absorbance at 753 nm could be observed (Figure 5). This clearly indicates that the presence of both the complex and the protein are simultaneously necessary to favor the formation of the DTB-SQ radical. The remarkable stability of this radical over a long time could be attributed to the formation of a stable $\text{LA-Cu}_2(\text{BDPAMHP})$ -DTB-SQ complex, by coordination of the DTB-SQ radical to the copper ions, that could thereafter get stabilized by protein residues.

This result is in line with the results already reported by the groups of DeGrado^[22] and Roelfes^[23] who showed that the 3,5-di-tert-butylsemiquinone (DTB-SQ) could be stabilized upon binding to the metal ions of the metal complexes inserted inside the binding cavities of respectively a de novo engineered metalloprotein $[\text{DFsc-Zn}(\text{II})_2]$ ^[24] and 2,2'-bipyridylalanine-Cu(II), -Fe(II), -Ni(II), and -Zn(II)-LmrR, artificial metalloproteins respectively.^[23]

Analyses of electron paramagnetic resonance (EPR) spectra that were recorded at room temperature made it possible to corroborate the formation of the paramagnetic radical in the presence of $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$ (Figure 6).

Indeed, whereas a weak signal around $g=2$ is observed in the presence of either CuSO_4 or $\text{Cu}_2(\text{BDPAMHP})$, the spectrum recorded in the presence of $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$ shows a signal characteristic of an organic radical ($g=2.0037$). This signal exhibits notable broadening (peak-to-peak linewidth of 9 Gauss) and does not exhibit hyperfine characteristics, thus suggesting immobilization of the radical inside the protein. This signal also corresponds to a radical species similar to that reported by the groups of DeGrado^[22] and Roelfes.^[23] Spin quantification shows a yield of $6.0 \pm 0.5\%$ radical formation in the presence of $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$. In the presence of CuSO_4 or $\text{Cu}_2(\text{BDPAMHP})$ or in the buffer control only a trace of radical formation ($\leq 0.3\%$ yield) could be observed.

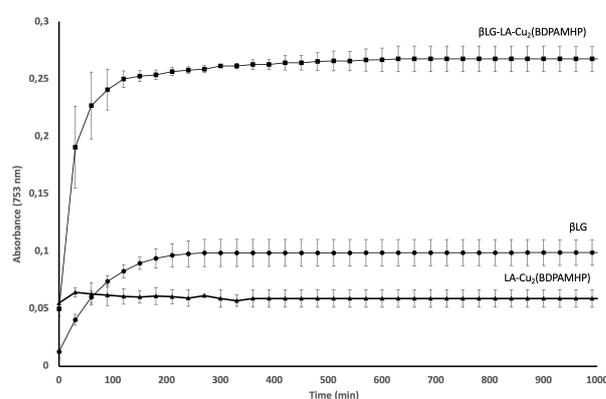


Figure 5. Evolution of the absorbance of DTB-SQ at 753 nm as a function of time, during the reaction of a 1/1 mixture of 0.5 mM DTBC and 0.5 mM DTBQ in the presence of 10 μM $\beta\text{LG-LA-Cu}_2(\text{BDPAMHP})$ (■), $\text{LA-Cu}_2(\text{BDPAMHP})$ (▲) or βLG (●) in 0.1 M Tris-HCl buffer, pH 7.5 containing 10% DMSO.

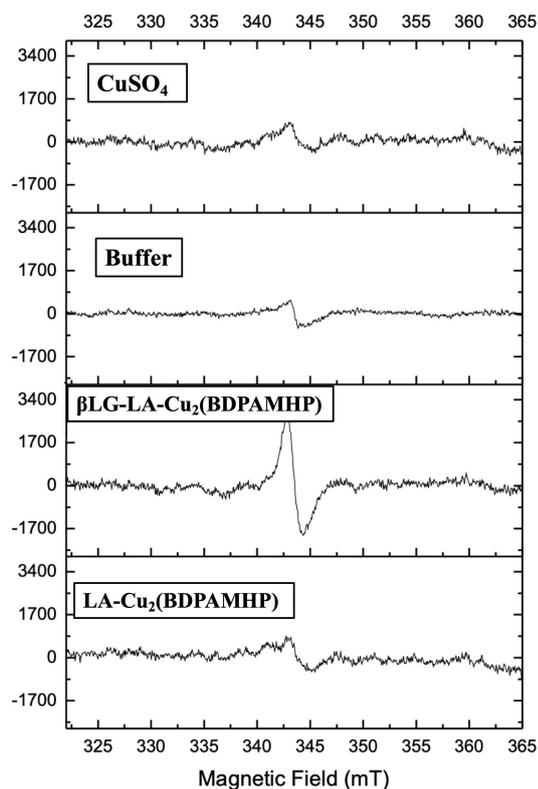


Figure 6. X-band EPR spectra of solutions a 1/1 mixture of DTBC and DTBQ 0.5 mM in DMSO in the presence of 10 μ M CuSO_4 , 0.1 M Tris-HCl buffer, pH 7.5, 10 μ M β LG-LA- Cu_2 (BDPAMHP), 10 μ M Cu_2 (BDPAMHP).

Results of the Molecular Docking

The molecular docking of LA- Cu_2 (BDPAMHP) in β LG provided three possible binding positions. All of these show the carbon chain of the lauric acid fragment bound in the pocket of the protein similarly to the original crystal structure, that of β LG-bound lauric acid. Indeed, the EF-hand at pH > 7 is in its open position providing access to the hydrophobic binding pocket for fatty acids and provides two crevices, one at each side of the hand. The copper complex fragment was found bound in one crevice in a first pose, projecting out of the other crevice in a second pose and bound on top of the main binding pocket of lauric acid in the third pose (Figure 7A). The first and third poses show good complementarities between the complex and the protein and scored best and second best, thus they were used for the subsequent molecular docking of DTB-SQ. The second pose scored lower as only the lauric acid fragment of LA- Cu_2 (BDPAMHP) interacted with the protein residues, therefore this binding was not considered for the subsequent molecular docking of DTB-SQ.

The binding of DTB-SQ on the copper complex on top of the main binding pocket of β LG did not show interactions with protein residues (Figure 7B). On the other hand, the binding of DTB-SQ on the copper complex bound in the side crevices showed good complementarities with proline 113 and alanine 111 and scored higher (Figure 7C). Such hydrophobic protein DTB-SQ interactions were indeed thought to be responsible for

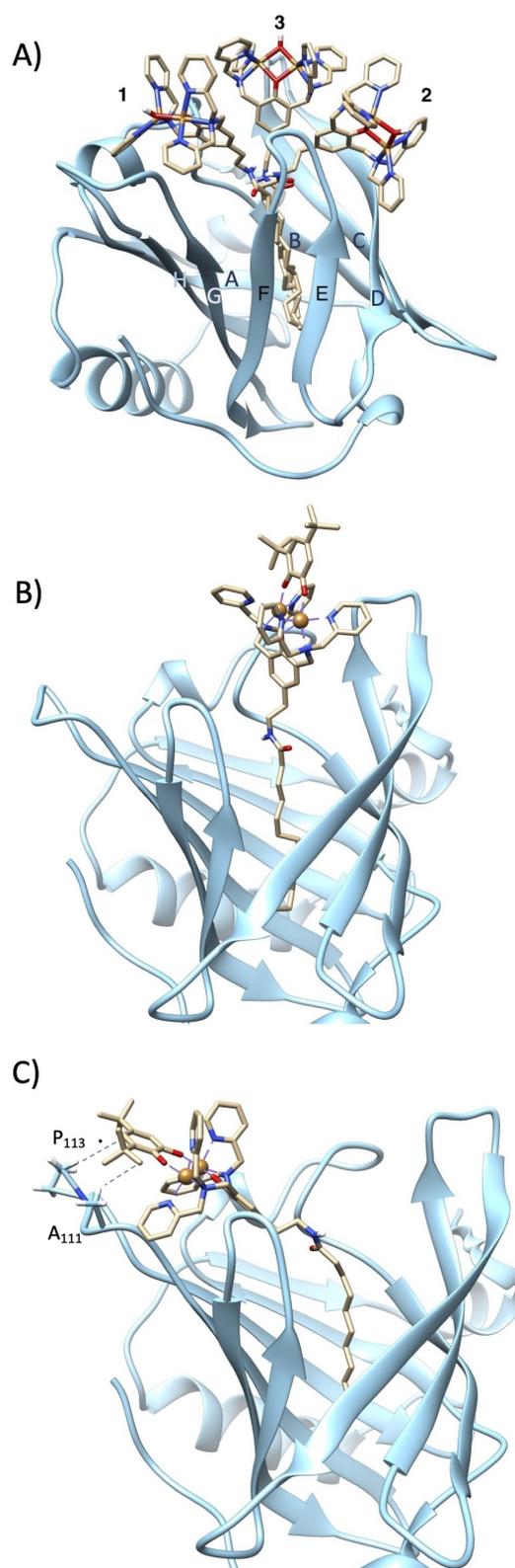


Figure 7. Molecular docking studies of the binding of LA- Cu_2 (BDPAMHP) and of the DTB-SQ radical inside β LG performed as described in the "Materials and Methods" section: A) the three possible binding positions of LA- Cu_2 (BDPAMHP) in β LG. The eight antiparallel β -sheets that form the symmetrical β -barrel cavity of β LG have been labeled A–H; binding of DTB-SQ on the LA- Cu_2 (BDPAMHP) copper complex bound B) on top of the main binding pocket of β LG; C) in the side one crevice of β LG.

stabilizing the radical.^[22,23] Consequently, the molecular docking results explain the experimentally observed radical stabilization by the biohybrid.

Conclusions

In conclusion, we exploited the affinity of beta-lactoglobulin (β LG for fatty acids to design an artificial binuclear copper metalloenzyme β LG-LA-Cu₂(BDPAMHP) which we expected to be capable of catalyzing the oxidation of catechol derivatives to quinones. The obtained biohybrid showed no catecholase activity, which could be due to the fact that it showed a certain capacity to bind and stabilize the intermediate semi-quinone radical DTB-SQ. Through optical and magnetic spectroscopic characterizations, we were indeed able to show that first, the copper complex played a key role in the binding of the semiquinone radical and, second, this stabilization could only occur in the protein pocket.

Materials and Methods

All commercially available reagents and solvents were purchased from Sigma-Aldrich, WWR, Carlo Erba and used without further purifications. β -Lactoglobulin A from bovine milk was purchased from Sigma-Aldrich.

Physical Measurements

UV-visible spectroscopy studies were performed on double beam CARY 300 BIO VARIAN spectrophotometer.

¹H NMR and ¹³C NMR spectra were recorded on BRUKER AM 360 and 400 spectrometers. Chemical shifts δ are given in ppm with respect to TMS signal, using CHCl₃ as an internal reference (δ 7.27 ppm/TMS). Electrospray ionization mass spectrometry experiments were carried on a MicrOTOF-Q Bruker.

The biohybrids were characterized by the quenching of the fluorescence of β LG performed on a CARY Eclipse spectrofluorometer.

¹H NMR spectra (400 MHz, CDCl₃) and ESI-MS spectra of compounds 1–4, UV-visible spectra and ESI-MS spectrum of complex 5, UV-visible spectra of complex the β LG-LA-Cu₂(BDPAMHP) biohybrid as well as those of the DTBC and DTQB substrates are presented in the supplementary information.

Preparation of Di-Copper(II)-N-(3,5-Bis((2,2'-Dipicolylaminomethyl)-4-Hydroxyphenethyl) Lauramide, LA-Cu₂(BDPAMHP) **5**

Di-copper(II)-N-(3,5-bis((2,2'-dipicolylaminomethyl)-4-hydroxyphenethyl) lauramide (LA-Cu₂DPAMHP) **5** was prepared in four synthetic steps as described in Scheme 1.

Tert-Butyl-(4-Hydroxyphenethyl)-Carbamate **1**

Tyramine (5 g, 28.8 mmol) was dissolved in dioxane (50 mL) and water (30 mL). Triethylamine (2.92 g, 28.8 mmol) and di-tert-butyl dicarbonate (6.27 g, 28.8 mmol) were then added. The solution was stirred at room temperature for 3 h. The reaction mixture was concentrated under reduced pressure. DCM was added and the organic phase was washed twice with a 5 % HCl solution. The organic phase was dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure. Product **1** was purified by column chromatography on a silica gel column, using EtOAc/DCM (1:9, v/v) as eluent, and was obtained as a clear oil (6.1 g, 90%). ¹H NMR (400 MHz, CDCl₃): δ = 7.04 (d, J = 8.5 Hz, 2H), 6.77 (d, J = 8.6 Hz, 2H), 4.53 (s, 1H), 3.33 (m, 2H), 2.71 (t, J = 7.1 Hz, 2H), 1.44 (s, 9H). ESI-MS m/z = 238.1428 (calculated 238.1437)

Tert-Butyl(3,5-bis((2,2'-Dipicolylaminomethyl)-4-Hydroxyphenethyl) Carbamate **2**

2,2'-dipicolylamine (DPA) (2.33 g, 11.67 mmol) was slowly added to a 37 % aqueous solution of formaldehyde (871 μ L, 11.67 mmol) and ethanol (3 mL). The reaction mixture was heated under reflux for 16 h and carbamate **1** (1 g, 4.21 mmol) in ethanol (9 mL) was added to the refluxing solution. The solution was heated under reflux for a further 5 days. The reaction mixture was cooled down and the solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed three times with a saturated aqueous solution of NaHCO₃. The organic phase was dried over Na₂SO₄, filtered and concentrated. Product **2** was purified by chromatography on a silica gel column, using DCM/MeOH (99.5/0.5, v/v) as the eluent, and was obtained as a brown oil (2.61 g, 94%). ¹H NMR (360 MHz, CDCl₃): δ = 8.55 (d, J = 4.5 Hz, 4H), 7.62 (td, J = 1.61 Hz, 4H), 7.51 (d, J = 7.7 Hz, 4H), 7.15 (t, J = 5.6 Hz, 4H), 7.05 (s, 2H), 5.99 (s, 1H), 3.95 (s, 8H), 3.87 (s, 4H), 3.33 (t, J = 7.0 Hz, 2H), 2.68 (t, J = 7.0 Hz, 2H), 1.42 (s, 9H). ESI-MS m/z = 660.3641 [M + H⁺] (calculated 659.8350).

4-(2-Aminoethyl)-3,5-bis(2,2'-Dipicolylaminomethyl)phenol **3**

TFA (4.45 mL, 58.2 mmol) was slowly added to a solution of **2** (1 g, 1.52 mmol) in DCM (40 mL). The solution was stirred at room temperature for 16 h under nitrogen, then the solvent was removed under reduced pressure. The residue was dissolved in DCM and washed three times with a saturated aqueous solution of NaHCO₃. The organic phase was then dried over anhydrous Na₂SO₄, filtered and concentrated to give the desired product **3** as an oil (823.4 mg, 97%). ¹H NMR (360 MHz, CDCl₃) δ = 8.51 (d, J = 4.9 Hz, 4H), 7.60 (t, J = 7.6 Hz, 4H), 7.57 (d, J = 7.8 Hz, 4H), 7.26 (t, J = 4.8 Hz, 4H), 7.11 (s, 2H), 3.86 (s, 8H), 3.77 (s, 4H), 2.93 (t, J = 7.4 Hz, 2H), 2.68 (t, J = 7.4 Hz, 2H). ESI-MS m/z = 560.3116 [M + H⁺] (calculated 560.3132).

N-(3,5-Bis((2,2'-Dipicolylaminomethyl)-4-Hydroxyphenethyl) Lauramide LA(BDPAMHP) **4**

Lauric acid (285 mg, 1.42 mmol) was dissolved in DCM (10 mL). 1,1'-carbonyldiimidazole (CDI) (178 mg, 1.1 mmol) was added. The reaction mixture was stirred at room temperature for 2 h and then was added slowly to a solution of **3** (700 mg, 1.25 mmol) and DMAP (13 mg, 0.1 mmol) in DCM (5 mL). After the solution was stirred at room temperature for 18 h, DCM (100 mL) and a saturated aqueous solution of NaHCO₃ were added to the reaction mixture. The organic phase was separated and washed three times with water and brine. The organic phase was dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column, using DCM/MeOH (99.5/0.5, v/v) as the eluent, to give product **4** as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ = 8.50 (d, J = 5.7 Hz, 4H), 7.60 (t, J = 7.6 Hz, 4H), 7.48 (d, J = 7.8 Hz, 4H), 7.12 (t, J = 6.1 Hz, 4H), 7.00 (s, 2H), 6.13 (t, J = 5.6 Hz, 1H), 3.87 (s, 8H), 3.78 (s, 4H), 3.47 (m, 2H), 2.70 (t, J = 6.6 Hz, 2H), 2.04 (m, 2H), 1.53 (m, 2H), 1.22 (m, 16H), 0.86 (m, 3H). ¹³C NMR (100 MHz, CDCl₃): δ = 173.4, 159.3, 154.7, 149.0, 136.7, 129.8, 128.7, 123.2, 122.2, 59.9, 55.0, 40.5, 36.9, 34.7, 32.0, 29.7, 29.6, 29.5, 26.0, 22.8, 14.3. ESI-MS *m/z* = 742.4767 (M⁺) (calculated 742.4803); 764.4586 (M + Na)⁺ (calculated 764.4622)

Synthesis of Di-Copper(II)-*N*-(3,5-bis((2,2'-Dipicolylaminomethyl)-4-Hydroxyphenethyl) Lauramide LA-Cu₂(BDPAMHP) **5**

An aqueous solution of copper (II) acetate (32.6 mg, 179.4 μmol) in water (5 mL) was added dropwise to a solution of **4** (60.5 mg, 81.53 μmol) in MeOH (30 mL). The solution was stirred at room temperature for 30 min. The solvent was evaporated (freeze dried) to give product **5** as a green solid (71 mg, 100%). ESI-MS *m/z* = 448.6687 (M²⁺) (calculated 448.6708); 956.3165 (M + AcO)⁺ (calculated 956.3555). UV-visible in CH₃CN: λ_{max}(nm) (ε(M⁻¹cm⁻¹)): 411 (756); 690 (125), in DMSO: λ_{max}(nm) (ε(M⁻¹cm⁻¹)): 407 (652); 679 (248)

Determination of the Dissociation Constant of the βLG-LA-Cu₂(BDPAMHP) Complex by Fluorescence Quenching

The interaction between βLG and LA-Cu₂(BDPAMHP) **5** was studied by intrinsic βLG fluorescence quenching upon successive additions of increasing amounts of LA-Cu₂(BDPAMHP) to the protein. Fluorescence measurements were performed on a CARY Eclipse spectrofluorimeter, using a 1 cm path length quartz cell. The spectra were recorded with a bandwidth of 5 nm for both excitation and emission beams at a scan rate of 250 nm.min⁻¹. Emission spectra of the protein Beta-Lactoglobulin were recorded between 300 and 400 nm with an excitation wavelength of 290 nm (Figure 2). Increasing amounts of a 0.3 mM solution of LA-Cu₂(BDPAMHP) in acetonitrile were added to a 5 μM solution of βLG in 20 mM HEPES buffer pH 7.5 in a final volume of 1 mL. The total added volume was inferior

to 10% of the total volume. The emitted fluorescence signal at 345 nm of the solution mixture (*F*) was measured after each addition of the complex **5**. *F*₀ represents the fluorescence signal of the protein in the absence of the ligand and *F*_{min} the fluorescence of the protein saturated with the ligand. Fluorescence values (Δ*F* = *F*₀ - *F*) were plotted as a function of the total ligand concentration ([L]_{tot}) in the solution and fitted with the following equation 1 using the Origin® program:

$$\Delta F = \Delta F_{\max} \frac{([n[P]_0 + [L]_0 + K_D] - \sqrt{([n[P]_0 + [L]_0 + K_D]^2 - 4n[P]_0[L]_0})}}{2n[P]_0} \quad (1)$$

where [P]₀ and [L]₀ are respectively, the total protein and ligand concentrations; *n* is the stoichiometry (number of ligand binding sites per protein); Δ*F*_{max} = *F*₀ - *F*_{min} represents the asymptotic value for Δ*F*; Δ*F*_{max} and *K*_D and *n* are the calculated values from the fitting equation.

Molecular Modeling

Modeling of the interaction between LA-Cu₂BPMP and βLG was obtained using the following method: molecular docking of LA-Cu₂BPMP in βLG was performed using the Schrödinger suite 2023-4. Firstly, the structure of the lauric acid chain was copied from that described in the crystal structure of its complex with βLG, which is available on the Protein Data Bank (PDB) website under the number 3UEU. It was then merged with the crystal structure of a similar Cu₂BPMP μ-hydroxo complex, which is available at the Cambridge Crystallographic Data Center (CCDC) under number 179943 using the command line entry "merge". This structure was chosen because the μ-hydroxo species was shown to be the major species in aqueous solution at 7 < pH < 9.^[24] Using the "3D Builder" toolbar window the two perchlorate counter ions in the crystalline mesh were deleted and the methoxy substituent of the ring was replaced by dimethyl amine and bound to lauric acid. The structure of the conjugate was optimized only for the newly created atoms and bonds using the molecular mechanics force field OPLS-2005^[26] in the "Optimization" window. The structure of the core of the copper complex and that of the carbon chain of lauric acid were thus derived from the corresponding crystal structures without major geometric change. "LigPrep" window was then used to generate: i) Epiks states and ii) soluble states at pH 7 (±2). Secondly, the βLG structure was prepared using the PDB 3UEU structure and following the "Protein Preparation Wizard" window interface: i) water molecules and salts at > 5 Å distance from protein residues were deleted; ii) by following the same procedure as for the ligand, the Epiks states and iii) the soluble states at pH 7 (±2) were generated; iv) the lauric acid present in the structure was also deleted and; v) its binding site was considered as the center of the grid space dedicated to molecular docking by following the "Receptor Grid Generation" interface tab. The "Ligand Docking" command line entry was then applied to the two partners and the top 2 scoring generated-structures were further used to dock DTB-SQ bound to the metal center. DTB-SQ was drawn using the "3D Builder"

toolbar window, which is compatible with unpaired electrons. "LigPrep" window was then used to i) generate Epiqs states and ii) soluble states at pH 7 (± 2). The "Receptor Grid Generation" interface tab was constrained to "Metal coordination" with both copper cations following literature.^[27]

Catalytic Activity and Kinetic Studies

All the kinetics measurements were carried out using Cary 60 UV-vis spectrophotometer from Agilent at 20 °C. The biohybrid formation results from a 4 hours incubation at 4 °C of a solution containing β -lactoglobulin (10.2 mg) dissolved in 950 μ L 0.1 M Tris-HCl buffer, pH 7.5 and 50 μ L of a 1 mM solution of LA-Cu₂(BDPAMHP) in MeCN, for a final concentration of about 50 μ M of the β LG-LA-Cu₂(BDPAMHP) AME.

The evaluation of the catecholase-like activity of the LA-Cu₂(BDPAMHP) complex and of the AME as well as the kinetic studies were performed spectrophotometrically under aerobic conditions by monitoring the oxidation of 3,5-di-tert-butylcatechol (DTBC) to 3,5-di-tert-butylquinone (DTBQ) at 400 nm as a function of time. The reactions were performed at 20 °C in a thermostated cell with 1 cm path length.

For catalytic activity studies, 10 μ M solutions of the LA-Cu₂(BDPAMHP) complex or of the AME in 0.1 M Tris-HCl buffer, pH 7.5 were treated at RT with 1 mM solutions of the substrate 3,5-di-tert-butylcatechol in MeCN (DTBC). The reaction progress was followed by UV/Vis. spectroscopy by monitoring the increase in the characteristic 3,5-di-tert-butylquinone (DTBQ) absorption band at 400 nm as a function of time. The data were taken at intervals of 0.05 min and the conversion was measured up to 10 min in each case.

Radical Stabilization Experiments

200 μ L of the β LG-LA-Cu₂(BDPAMHP) AME 50 μ M in 0.1 M Tris-HCl buffer, pH 7.5 were added to 700 μ L of 0.1 M Tris-HCl buffer, pH 7.5 followed by the addition of 100 μ L of a 1/1 mixture of DTBC and DTBQ, 5 mM in DMSO. UV-visible spectra were recorded during 18 hours (1 scan every 30 min).

EPR Spectroscopy

X-band EPR spectra were recorded on a Bruker ELEXSYS 500 spectrometer equipped with a Bruker ER 4116DM X band resonator, an Oxford Instrument continuous flow ESR 900 cryostat, and an Oxford ITC 503 temperature control system under the following conditions: T = 298 K, MW freq. = 9.6351 GHz, MW power = 12 mW, MA = 8 Gauss, Mod. Freq = 100 KHz, Gain = 56 db, 18 scans. 200 μ L of the β LG-LA-Cu₂(BDPAMHP) AME 50 μ M solution in 0.1 M Tris-HCl buffer, pH 7.5, was added to 700 μ L of 0.1 M Tris-HCl buffer, pH 7.5, followed by the addition of 100 μ L of a 1:1 mixture of DTBC and DTBQ, both at a concentration of 5 mM in DMSO. After 1 hour, 100 μ L of this solution mixture was transferred to a capillary.

Quantification of the radical species was made by comparing the double integral of the EPR signal at $g = 2.0037$ with those obtained in a calibration curve of TEMPO at concentrations 1.48, 3.70 and 7.40 μ M under the same experimental conditions. A concentration of 600 ± 3 nM was found for the DTB-SQ radical.

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Conflict of Interests

The authors declare no conflict of interest.

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