

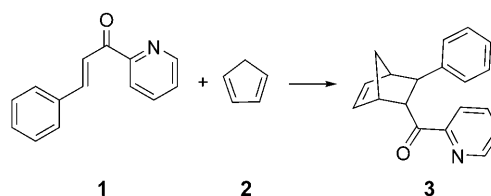
An Artificial Metalloenzyme: Creation of a Designed Copper Binding Site in a Thermostable Protein**

John Podtetenieff, Andreas Taglieber, Eckhard Bill, Edward J. Reijerse, and Manfred T. Reetz*

During the last three decades different types of synthetic metalloenzymes have been prepared, including those based on anchoring appropriate ligands such as diphosphines, phthalocyanines, or dipyrrolyl moieties covalently or non-covalently onto proteins.^[1,2] The respective transition-metal complexes constitute hybrid catalysts that mediate reactions such as asymmetric rhodium-mediated olefin hydrogenation, Diels–Alder cycloadditions, and thioether sulfoxidation.^[1] The bioconjugative process produces a single catalyst, which may or may not lead to high enantioselectivity. To address this fundamental problem, we proposed some time ago the idea of tuning such hybrid catalysts by the genetic methods of directed evolution.^[3] It was recently implemented experimentally in a proof-of-concept study^[4] using the system reported by Wilson and Whitesides^[5,6] which is based on avidin (or streptavidin) and a biotinylated diphosphine complexed to rhodium.^[7] In laboratory evolution of this hybrid catalyst, the enantioselectivity of the hydrogenation of olefins was increased stepwise from 23 % *ee* to 65 % *ee* after three rounds of mutagenesis/screening.^[4] However, this study suffered from several practical drawbacks, which have to do with the prerequisites inherent in our Darwinian approach to catalyst optimization: 1) An efficient expression system of the host protein is required; 2) The host protein should be robust (thermostable); 3) Separation of the host from other proteins present in the supernatant needs to be efficient; 4) Bioconjugation should be essentially quantitative. Although covalent and noncovalent anchoring of ligand/metal moieties to robust proteins and subsequent tuning utilizing directed evolution remains a promising concept,^[3,7] we now present an alternative approach which offers some advantages.

In the present study a transition-metal binding site is created by introducing coordinating amino acids at geometrically appropriate positions in a robust host protein, an

experimental process which is easily performed using conventional site-specific mutagenesis.^[8] The respective residues of the mutated protein themselves serve as the metal-binding ligand,^[9] eliminating the usual bioconjugation and purification steps.^[7] As the host protein we chose the synthase subunit of imidazole glycerol phosphate synthase from *Thermotoga maritima*, tHisF, which is an unusually thermostable enzyme that is essential in the biosynthesis of histidine.^[10] This robust protein can be expressed efficiently in *E. coli*; purification on a large scale^[11] or in the wells of microtiter plates^[12] is possible by using a simple heat treatment which leads to the denaturation and precipitation of all other proteins in the cell free extract. It is therefore an ideal protein host, as shown previously in the case of covalent anchoring of ligands through conventional bioconjugation.^[12] The X-ray structure of tHisF reveals a typical TIM-barrel structure eightfold α/β motif having a narrow “bottom” and a wide “top”, both of which are open.^[13] The latter was chosen for constructing the metal-binding motif. Indeed, the top of the barrel widens to a relatively large cleft that appears to be not only ideal for introducing coordinating amino acids, but also for performing organic reactions at the respective transition-metal site. We chose Cu^{II} as the metal, envisioning the newly designed metalloenzyme as a catalyst in the asymmetric Diels–Alder reaction of azachalcone **1** and cyclopentadiene (**2**) for formation of cycloadduct **3** (Scheme 1).



Scheme 1. Model Diels–Alder reaction in aqueous medium.

It is well known that Cu^{II} complexes of chiral synthetic ligands catalyze a variety of asymmetric Diels–Alder reactions efficiently in organic solvents.^[14] Moreover, the model reaction used here has previously been performed with Cu^{II} complexes of amino acids,^[15] Cu^{II} DNA intercalating agents,^[16] and Cu^{II}–phthalocyanine anchored noncovalently to serum albumins,^[17] all in aqueous medium.

To design an appropriate Cu^{II} binding site in the robust host tHisF, we were guided by nature. The various types of copper proteins usually coordinate the metal through a combination of nitrogen and oxygen (or sulfur) ligands. The simplest motifs are found in the type 2 copper center of the trinuclear copper proteins such as ascorbate oxidase, which is

[*] Dr. J. Podtetenieff,^[14] Dr. A. Taglieber,^[14] Prof. Dr. M. T. Reetz
Max-Planck-Institut für Kohlenforschung
Kaiser-Wilhelm-Platz 1, 45470 Mülheim an der Ruhr (Germany)
Fax: (+49) 208-306-2985
E-mail: reetz@mpi-muelheim.mpg.de

Dr. E. Bill, Dr. E. J. Reijerse
Max-Planck-Institut für Bioanorganische Chemie
45470 Mülheim an der Ruhr (Germany)

[*] These authors contributed equally to this work.

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coordinated by two nitrogen ligands from the imidazole moiety of histidine, and one or more oxygen ligands (water).^[18] The above information along with careful analysis of the tHisF structure and the presence of an existing aspartate residue at position 11 led us to consider engineering a 2-His-1-carboxylate motif (His/His/Asp) at the top of the TIM barrel. The coordinating His/His/Asp triad has been shown to occur in Fe^{II} metalloenzymes which operate by a different mechanism.^[19] The crystal structure of tHisF suggested that the β strand neighboring Asp11, with leucine and isoleucine at positions 50 and 52, respectively, would ideally position the two histidine side chains in three-dimensional space (within ca. 4–5 Å). The designed putative metal-binding site comprising Asp11/His50/His52 near the top rim of tHisF is shown in Figure 1. We anticipated preferential Cu^{II} binding by these three donor moieties, augmented by water molecules acting as ligands.

Since a cysteine occurs further down in the barrel at amino acid position 9, which could compete for metal binding, mutation Cys9Ala was also carried out. In a stepwise process, standard site-specific mutagenesis (QuikChange)^[8a] was applied to convert WT tHisF into mutant Cys9Ala/Leu50His/Ile52His. Subsequently it was used to bind Cu^{II}, thereby forming an artificial metalloenzyme, which served as a catalyst in the model Diels–Alder reaction occurring in aqueous medium. Exploratory experiments proved to be encouraging. We observed an *ee* value of 35 % for the favored *endo* product **3** (*endo/exo* = 14:1) and a reaction rate higher than that of the reaction catalyzed by WT Cu^{II}/tHisF which induces minimal enantioselectivity (3 % *ee*).^[20]

Nevertheless, we realized that WT tHisF contains four additional histidines scattered across the surface of the protein at amino acid positions 84, 209, 228, and 244, all of which might compete for Cu^{II}, causing problems with the characterization of the Cu^{II} complex and ambiguity in the interpretation of the model reaction. Therefore, alanine was introduced at these positions, while retaining the previous amino acid substitutions. The new mutant was designated as HHD-4xala. After verifying that these amino acid substitutions do not impair expression and folding, a negative control mutant was similarly prepared, namely one in which the WT amino acids at positions 50 and 52 were retained as leucine and isoleucine, respectively, but the four surface histidines at positions 84, 209, 228, and 244 were replaced by alanine; this mutant was designated as NC-4xala. Thereafter, the Cu^{II}-catalyzed model Diels–Alder reaction was again studied (Table 1). Notable enantioselectivity is possible only with the hybrid catalyst Cu^{II}/HHD-4xala (46 % *ee*). Moreover, this catalyst leads to an enhanced reaction rate as indicated by the higher conversion relative to the other (control) reactions. The results strongly support our conjecture that Cu^{II} is indeed coordinating to the putative binding site in Cu^{II}/HHD-4xala, certainly to a large extent. Moreover, we performed yet another control experiment in which aspartate at position 11 of HHD-4xala was mutated to Ala11 (designated as HHA-4xala), the respective Cu^{II} complex leading to only 56 % conversion under standard reaction conditions, the *ee* value being a mere (4 ± 0.9) % (*endo/exo* = 8:1; Table 1). This also speaks for selective complexation of Cu^{II} by HHD-4xala at

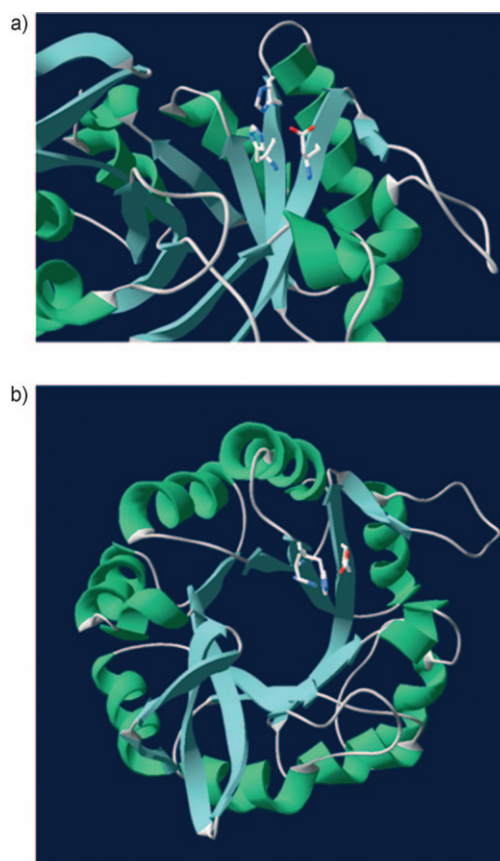


Figure 1. Designed putative metal binding site in the thermostable protein tHisF (based on the crystal structure).^[13] a) Close-up view of the designed binding site. b) Top view of modified tHisF with binding site.

Table 1: Catalytic Diels–Alder reaction^[a] of **1** and **2** with formation of **3** in water.

Catalyst	Conversion [%]	<i>ee</i> [%]	<i>endo/exo</i>
Cu ^{II} /HHD-4xala	73 ± 1.6	46 ± 0.5	13:1
Cu ^{II} /NC-4xala	61 ± 0.5	5 ± 0.1	9:1
Cu ^{II} /HHA-4xala	56 ± 1.2	4 ± 0.9	8:1
free Cu ^{II}	44 ± 2.9	1 ± 0.3	7:1
no catalyst (buffer)	26 ± 3.1	1.5 ± 0.5	8:1

[a] Reactions were performed in a total volume of 1 mL at the following final concentrations: 153 μ M tHisF, 115 μ M CuSO₄, 1.05 mM azachalcone **1**, and 5.25 mM Cp **2**. The concentrations result in a catalyst loading of 11 % (total Cu^{II}), and a Cu^{II}/tHisF ratio of 75 %. (reaction time: 96 h). All experiments were carried out at least twice and in triplicate.

the expected metal-binding site Asp11/His50/His52. However, since the crystal structure could not be obtained thus far, additional evidence for our postulate was needed.

To demonstrate selective Cu^{II} complexation at the designed binding site, we turned to electron paramagnetic resonance spectroscopy (EPR). It is well known that EPR can be used to characterize the nature of the ligand environment in Cu^{II} complexes.^[21] Initially, we recorded spectra of both catalyst systems, Cu^{II}/HHD-4xala and Cu^{II}/NC-4xala using

continuous wave X-band instruments with frozen samples (Figure 2).

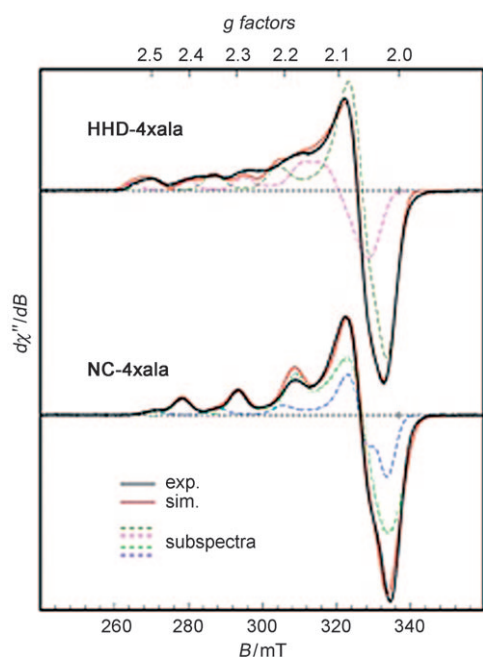


Figure 2. EPR spectra of catalysts Cu^{II} /HHD-4xala and Cu^{II} /NC-4xala recorded at 25 K (microwave frequency 9.427056 GHz, power 0.2 mW, modulation 1 mT/100 kHz), black lines: experimental spectra, red lines: simulations with two subspectra, dotted lines: subspectra with intensity ratios 36:64 (HHD-4xala) and 75:25 (NC-4xala).

The results clearly indicate a difference in the coordination of Cu^{II} between the two mutants. Both spectra can best be fitted with two subspectra arising from very similar Cu environments. None of the components found for HHD-4xala, however, coincide with the subspectra of the control NC-4xala (see anisotropic g values and magnetic hyperfine coupling in Table 2). The presence of subspectra, in both cases, reveals some conformational heterogeneity of the Cu^{II} resulting from the flexibility of the ligand arrangements. Moreover, the EPR spectrum of Cu^{II} /NC-4xala, which deviates from that of Cu^{II} in buffer (not shown), indicates that the negative control has a copper binding site somewhere on its surface. Indeed, proteins commonly have nonspecific

copper binding sites on their surfaces. The spectrum of the Cu^{II} complex of the HHD-4xala mutant, in contrast, has a significantly larger g anisotropy and different hyperfine coupling compared to the control catalyst Cu^{II} /NC-4xala (Table 2). This data lies in a range that is characteristic for type 2 copper centers which are known to be coordinated by histidine.^[22] Therefore, in the presence of the protein containing the designed binding triad (mutant HHD-4xala), Cu^{II} clearly shifts to a new coordination site, resulting in a different spectrum that is indicative of histidine coordinated to Cu^{II} . To additionally characterize this artificial site, the magnetic interactions of the ^{14}N nuclei in the Cu^{II} coordinating ligands were monitored using hyperfine sublevel correlation (HYSCORE) spectroscopy, which is a two-dimensional variant of electron spin envelope modulation (ESEEM) spectroscopy.^[23]

HYSCORE experiments on Cu^{II} /HHD-4xala taken at the $g_{\perp}$ position of the EPR spectrum (3300 Gauss) show strong, broad correlation features at (1.5, 3.5) GHz. As shown in Figure 3, these line positions are very characteristic for histidine coordinated to Cu^{II} and represent the so-called 1–3 transitions of ^{14}N nuclear spins of the noncoordinating imidazole nitrogen atoms.^[24] By using spectral simulation, a good estimate of the nuclear quadrupole interaction (NQI) and hyperfine (HFI) parameters can be obtained from just the observed 1–3 transitions in the HYSCORE spectra. The NQI values of ^{14}N are very characteristic for its chemical environment and can be used to identify the type of nitrogen ligand. The HYSCORE spectrum of Cu^{II} /HHD-4xala was simulated

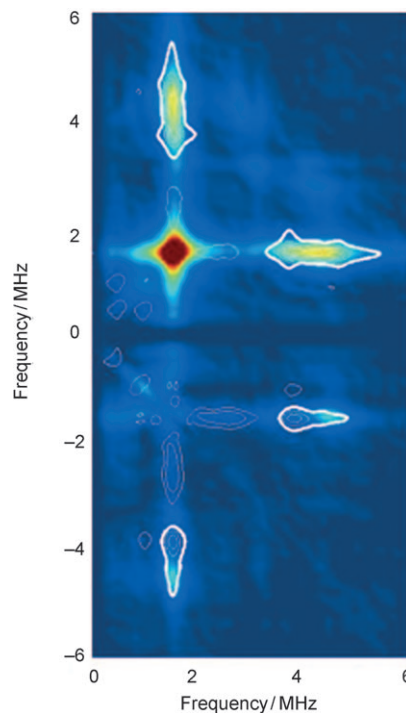


Figure 3. HYSCORE spectrum density plot of Cu^{II} /HHD-4xala recorded at the $g_{\perp}$ field position (3300 Gauss) and time parameter $\tau = 120$ ns. Spectral simulations are shown as contour lines. ^{14}N coupling parameters: $A = (2.2, 1.5, 1.2)$ MHz, $K = 0.39$ MHz, $\eta = 0.7$. The diagonal features originate from pulse imperfections in the experiment^[23] and are not reproduced in the simulation.

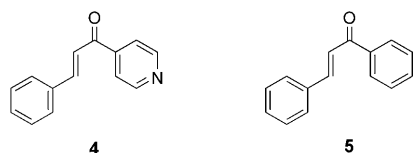
Table 2: EPR anisotropic g_1 and g_2 values and magnetic hyperfine coupling tensors (A_1 and A_2) of both subspecies (S1 and S2) found in Cu^{II} /HHD-4xala and Cu^{II} /NC-4xala.^[a]

Subspectra	Cu^{II} /HHD-4xala	Cu^{II} /NC-4xala
$g_1^{\text{[a]}}$	[2.058, 2.119, 2.343]	[2.096, 2.027, 2.236]
$ A_1 ^{\text{[b]}}/10^{-4} \text{ cm}^{-1}$	[–, –, 165.] ^[c]	[–, –, 159.] ^[c]
$g_2^{\text{[a]}}$	[2.070, 2.046, 2.280]	[2.057, 2.069, 2.269]
$ A_2 ^{\text{[b]}}/10^{-4} \text{ cm}^{-1}$	[–, –, 184.] ^[c]	[–, –, 185.] ^[c]
Rel. intensity S1/S2	36:64	75:25

[a] Anisotropic g values. [b] Components of the magnetic hyperfine coupling tensor for the interaction with $63/65\text{Cu}$ nuclei ($I = 3/2$). [c] Dashes indicate undetermined values.

assuming a large asymmetry parameter η . The obtained coupling parameters $A = (2.2, 1.5, 1.2)$ MHz, $K = 0.39$ MHz,^[25] and $\eta = 0.7$ are quite common for a remote imidazole nitrogen atom in histidine coordinated Cu^{II} complexes,^[26] which is in line with Cu^{II} coordination at our designed binding site. In contrast, the ¹⁴N magnetic coupling parameters determined for the copper binding site in the Cu^{II} complex of the negative control mutant catalyst, Cu^{II}/NC4xala: $A = (1.2, 1.0, 0.8)$ MHz, $K = 0.87$ MHz and $\eta = 0$, are typical for binding to a peptide backbone (amido) nitrogen atom.^[26b]

In the present work and in all previous studies^[15–17] regarding transition-metal-catalyzed Diels–Alder reactions of azachalcone **1**, it has been assumed that the lone electron pairs at the carbonyl O atom and the pyridine N atom form a five-membered chelated structure with Cu^{II}, thereby activating the substrate as a result of lowering the energy of the lowest occupied molecular orbital (LUMO). To test this hypothesis, we subjected chalcones **4** and **5** to the Diels–Alder



reaction using the artificial metalloenzyme Cu^{II}/HHD-4xala. Since these substrates cannot form a chelate, we expected results very different from those arising from the reaction of **1**. Indeed, the respective Diels–Alder reactions occurred sluggishly and the respective cycloadducts were obtained in less than 3% yield. These observations support our hypothesis regarding the mechanism of activation by Cu^{II}/HHD-4xala.

In conclusion, we have succeeded in establishing a new route to synthetic metalloenzymes by utilizing site-specific mutagenesis to create a Cu^{II} binding motif in a thermostable protein. The initial assumption that Cu^{II} does in fact coordinate at the designed Asp/His/His binding site was supported by the observation of notable enantioselectivity, enhanced *endo*-selectivity, and somewhat higher reaction rate of the model Diels–Alder cycloaddition. Systematic mutation studies utilizing site-directed mutagenesis as part of the control experiments, and especially extensive EPR studies corroborated this conclusion. The CW EPR measurements show that Cu^{II} coordination is distinctly different in copper complexes of the designed hybrid catalyst Cu^{II}/HHD-4xala as compared to those of the negative control Cu^{II}/NC-4xala. HYSCORE experiments specifically show that the metal is directly coordinated to an imidazole moiety (histidine) in the designed mutant HHD-4xala, and that such coordination does not occur in the case of the control mutant NC-4xala. Since the only source of imidazole ligands in the tHisF mutant HHD-4xala are the two histidine residues at positions 50 and 52, it is clear that copper is coordinated at the artificial binding site, where the asymmetric Diels–Alder reaction occurs. Future work will focus on laboratory evolution to enhance both enantioselectivity and activity, including the use of other transition metals at the same designed binding site

allowing for the creation of a family of new artificial metalloenzymes.

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