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Short Self-Assembling Peptides Are Able to Bind to Copper and Activate Oxygen

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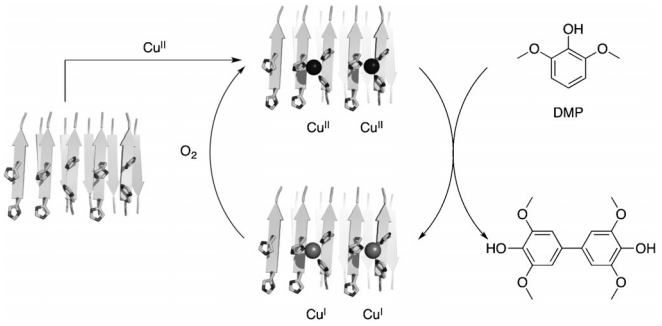
Abstract: *We have shown that de novo designed peptides self-assemble in the presence of copper to create supramolecular assemblies capable of carrying out the oxidation of dimethoxyphenol in the presence of dioxygen. Formation of the supramolecular assembly, which is akin to a protein fold, is critical for productive catalysis since peptides possessing the same functional groups but lacking the ability to self-assemble do not catalyze substrate oxidation. The ease with which we have discovered robust and productive oxygen activation catalysts suggests that these prion-like assemblies might have served as intermediates in the evolution of enzymatic function and opens the path for the development of new catalyst nanomaterials.*

Proteins extensively rely on metal cofactors to achieve structural stability and function. Indeed, it is estimated that up to a third of all proteins contain various metal ions.[1] Metalloenzymes are capable of efficiently tuning the properties of a metal ion to catalyze very difficult chemical transformations such as the conversion of methane into methanol or the oxidative depolymerization of lignin using simple oxidants such as dioxygen and hydrogen peroxide.[2] Recently, we reported short peptides designed from first principles that self-assemble in the presence of zinc to form amyloid-like fibrils, which efficiently catalyze the hydrolysis of *p*-nitrophenyl esters.[3] The most catalytically active fibrils showed activity that rivals that of the natural enzymes by weight. The ease with which we were able to discover such activity—the original library included only 10 peptides—suggests that the self-assembly of short peptides could have been a major pathway in enzyme evolution and opens the door to utilizing this approach to a large array of chemical reactions. In this paper we ask whether self-assembly of peptides can be used to discover supramolecular catalysts for oxygen activation.

Designing efficient catalysts for oxygen activation presents several challenges: 1) the ligand in the primary coordination sphere needs to properly tune the redox potential of the metal ion; 2) the ligand has to be able to accommodate different oxidation states of the metal ion; 3) catalysis should not involve the formation of reactive oxygen species that can promote ligand decomposition. Thus it is hardly surprising that only a handful of successful protein or peptide designs capable of promoting redox reactions have been reported to date.[4]

The very simple coordination sphere produced by the self-assembly of peptides previously reported by us[3] could potentially accommodate metal ions other than zinc. We set out to develop a copper based self-assembling system since copper ions are commonly employed in redox-active enzymes, are hydrolytically stable at near-neutral pH, and provide convenient spectroscopic handles.

As a model reaction in our studies, we chose the oxidation of 2,6-dimethoxyphenol (DMP, Scheme 1). Oxidative dimerization of DMP produces a colored product that can be easily assayed in a high-throughput fashion. This reaction proceeds through Cu^{II}-mediated oxidation of the substrate; it is well benchmarked and has been extensively mechanistically characterized.[5] Additionally, the oxidation of substituted phenols is commonly used in the polymer industry.[6]



Scheme 1. Copper-mediated oxidation of dimethoxyphenol (DMP) by dioxygen. The fibril structures shown are not derived from experimental data; they are based on a previously reported computational model for zinc-containing peptide assemblies.[3].

To test whether self-assembling peptides can indeed support copper-mediated substrate oxidation, we used a small focused library of peptides previously shown to self-assemble in the presence of zinc and catalyze ester hydrolysis. The results of the initial screening are presented in Figure 1. The most active peptide (**11**, Ac-IHIHIQI-$CONH_2$) showed activity that was more than an order of magnitude higher than baseline activity. The levels of activity are highly sequence dependent: the non-fibril-forming control peptide (**14**, NH_2-IHIHIQI-COOH) that has the same primary sequence but lacks the caps on the termini shows activity that is lower than that of free copper ions in buffer. As in our previous work, peptides containing residues that promote β-strand secondary

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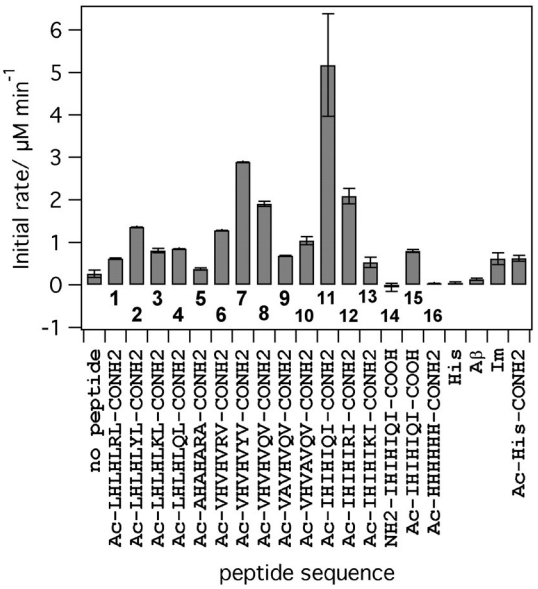

Figure 1. Initial rates of product formation catalyzed by Cu^{II} (10 μM) in the absence/presence of various peptides (20 μM). All reactions are in 25 mM HEPES buffer pH 7.9 at room temperature with DMP (500 μM). His = histidine (40 μM), Im = imidazole (40 μM), Ac-His-$CONH_2$ = *N*-acetyl histidine amide (40 μM), Aβ = amyloid beta (10 μM).

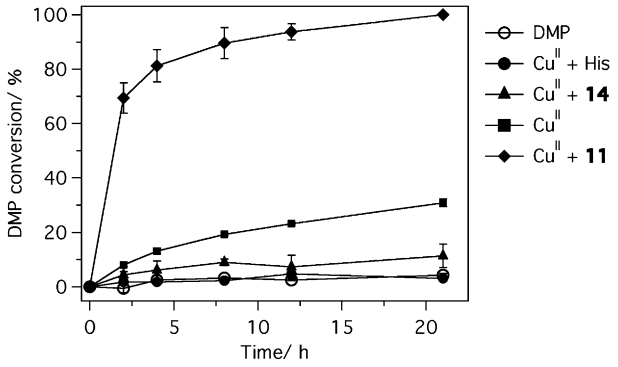

Figure 2. Conversion of dimethoxyphenol as catalyzed by Cu^{II} in buffer or Cu^{II} in the presence of histidine (His), peptide **11**, or peptide **14**. The reaction was initiated by adding DMP (200 μM). Aliquots were taken at various time points and immediately analyzed by HPLC. Reaction conditions: 25 mM HEPES buffer pH 8, Cu^{II} (40 μM), and peptide (80 μM) or His (160 μM).

structure (Ile and Val; **7**, **8**, **11** and **12**) showed high activity compared to peptides with residues with a lower propensity to form β-sheets (Leu and Ala; **1**–**5**). Circular dichroism (CD) spectra of **7** and **11** showed clear β-sheet character in the presence of copper (Figure S1 in the Supporting Information). Replacing either His2 or His4 with alanine significantly reduces the activity (Figure 1, peptides **9**, **10**). While both **9** and **10** form large aggregates with β-sheet character and bind copper, albeit with different stoichiometry (Figures S2, S3), they demonstrate lower activity under all conditions. This suggests that the His-X-His motif provides the optimal functional-group arrangement among the peptides studied in this work. The nature of the residue in position 6 is also important for catalytic activity, with peptides containing Gln and Tyr in this position producing the most active catalysts. Under the same conditions, amyloid beta peptide (Aβ 1–40), which is known to self-assemble and bind copper,[7] does not catalyze DMP oxidation. The self-assembly of histidine-containing peptides alone is thus not sufficient for catalysis.

We chose **11**, the most active peptide, for in-depth characterization. Based on the Job's plot of activity (Figure S3) and copper titrations (Figure S4), we determined the Cu^{II}/**11** ratio in the complex to be approximately 1:2. The rate enhancement of DMP oxidation relative to Cu^{II} in buffer is pH-dependent and reaches more than 65-fold at pH 6 (Figure S5). The catalyst undergoes multiple turnovers, resulting in complete oxidation of the substrate in less than 24 hours (Figure 2). The histidine complex of copper is completely inactive; imidazole and *N*-acetyl histidine amide show low activity, on a par with that of the non-self-assembling peptides **4** and **5** under the same conditions (Figure 1 and Figure S6). The non-self-assembling hexahistidine peptide **16** (Figure S2) is also completely inactive, thus suggesting that multivalent coordination by histidine residues is not sufficient for activity. Peptide **11** must thus provide an appropriate coordination sphere for the copper to engage in productive catalysis. Next, we performed ultracentrifugation experiment to determine the nature of the active species. Centrifugation for 1 hour at 100 000 *g* completely removes peptide **11** species from the solution, as concluded from the absorbance of the supernatant (Figure S7). The supernatant shows no activity in DMP oxidation, thus suggesting that large peptide aggregates are responsible for catalysis. Similarly, filtration of the copper/peptide complex through a 0.22 μm membrane (Figure S8) produces filtrate that has no activity. On the other hand, dialysis of the peptide solution fully preserves its oxygen activating activity (Figure S9). The inactive peptide **14** bound to copper, as shown by the EPR data discussed below, did not show any secondary structure (Figures S1, S6) and did not precipitate in ultracentrifugation experiments under the same conditions (Figure S10).

Ultracentrifugation also provided us with an opportunity to quantitatively characterize the binding of copper to the fibrils. Measurement of the equilibrium concentrations of copper in the supernatant after centrifugation showed that under the conditions used, approximately 90 % of the copper is bound by the peptide (Figure S10).

To probe the Cu^{II} coordination environment in different peptides, we used low-temperature EPR spectroscopy. The g_{II} and A_{II} values for Cu^{II} are commonly used to determine the composition of the copper coordination sphere. EPR spectra of Cu^{II} bound to the most active peptides (Ac-IHIHIQI-$CONH_2$ and Ac-VHVHVYV-$CONH_2$) are of the classic type 2 with $g_{II} = 2.27$ and $A_{II} = 167$ G, values that are consistent with either an 3N1O or 2N2O coordination environment in the equatorial plane based on the Blumberg–Peisach plots (Figures 3 and Figure S11).[8] Peptide **14** (NH_2-IHIHIQI-COOH), which does not aggregate, has no β-sheet structure at pH 8 as shown by CD spectroscopy and ultracentrifugation (Figures S6 and S10), and is inactive in the DMP oxidation assay, gives a distinctly different EPR spectrum (Figure S11). These results suggest a major change

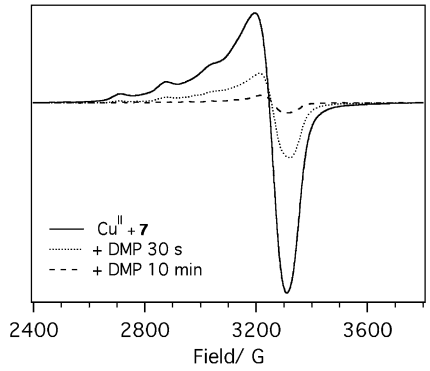

Figure 3. X-band EPR spectra of Cu^{II} (150 μM) in the presence of peptide **7** (300 μM) acquired at pH 7.6 in buffer (25 mM HEPES, 10% glycerol) at 10 K. Frequency 9.39 GHz, power 5.024 mW, modulation frequency 100 kHz, modulation amplitude 8 G, conversion time 40 ms, time constant 163.8 ms. After acquiring the spectrum (solid line), DMP was added to the sample to a final concentration of 1 mM (5 μL of 70 mM solution) and the sample was mixed and frozen (+DMP, 30 s, dotted line). After recording another EPR spectrum, the sample was thawed and incubated at room temperature for 10 min (+DMP, 10 min, dashed line).

in the coordination environment of the copper despite an essentially identical primary peptide sequence. The EPR spectra also shed light on the possible reaction mechanism. Addition of DMP to Cu^{II}/**7** results in a gradual diminishing of the EPR signal (Figure 3), which is consistent with the reduction of Cu^{II} to Cu^{I} and is in line with the previously proposed mechanism.[5c,d]

The contribution of self-assembly to the overall activity can be examined by comparing the properties of peptides **4**, **5**, and **11**. The identities of the metal-binding residues are the same in all cases but the residues in the "hydrophobic" positions are varied. Peptides **4** and **5**, which have a lower β-sheet-forming propensity bind copper but show neither aggregation nor β-sheet character in the presence of the metal ion (Figures S6, S7, S10). The copper coordination environments in **5** and **11** are different as judged from the EPR data (Figures S11, S12). The observed differences in activity can be explained by the high stability of the β-sheet assemblies that lock the histidine residues into a conformation that is more suitable for appropriate copper coordination, in a manner akin to the way a protein fold modifies the properties of bound metal ions. A comparison of peptides **4** and **11** is particularly instructive. Isomerization of the side chain of an amino acid residue that is not involved in the primary coordination sphere of the copper ion (leucine to isoleucine) leads to improved self-assembly and an approximately 5-fold increase in the initial rate of phenol oxidation.

We established that the oxidation of DMP as promoted by the Cu^{II}/peptide complex is oxygen-dependent, since removal of oxygen prevented formation of the product but introduction of O_2 into the reaction mixture immediately resulted in DMP oxidation (Figure S13). Oxygen, superoxide, and H_2O_2 alone do not oxidize DMP over a timescale of several hours, however formation of the product occurs immediately upon mixing the Cu^{II}/peptide complex and DMP, thus emphasizing the crucial role of Cu^{II} in this reaction. Addition of catalase (Figure S14) or superoxide dismutase (Figure S15) does not substantially diminish the reaction rate, thus mechanistically supporting Cu^{II}-promoted oxidation and effectively excluding the possibility of radical oxidation of the substrate. Among the different metal ions tested (Cu^{II}, Fe^{III}, Fe^{II}, Mn^{II}, Ni^{II}, Co^{II}), only Cu^{II} catalyzes DMP oxidation to any appreciable extent, thus suggesting that the redox potential of Cu^{II} and the coordination environment supported by the fibril core are optimal for catalysis (Figure S16).

Supramolecular approaches to designing efficient catalysts for chemical reactions have been extremely productive.[9] The self-assembly properties of peptides have been previously used to create multidentate ligands for transition-metal catalysts,[10] hydrogels with esterase activities,[11] and light-capturing materials.[12] The ability to genetically encode large peptide libraries opens the path for the discovery and optimization of peptide catalysts by using high-throughput techniques.[13] Herein, we show that the self-assembly of short de novo designed peptides results in the formation of efficient supramolecular catalysts that are capable of oxygen activation. Moreover, we show that supramolecular assemblies are capable of supporting oxygenation catalysis that does not rely on radicals. This finding underscores the fact that amyloid-like assemblies formed by even very short peptides can facilitate various chemical transformations in a highly sequence-specific manner. Considering recent findings that amyloid-supported metal sequestration and catalysis is more likely to be the rule than an exception,[3,14] we expect de novo designed self-assembled catalytic peptides to combine the highly controlled metal coordination sphere common in homogeneous catalysis with the practical advantages of heterogeneous catalysts. Moreover, synergistic interactions observed in these systems provide additional opportunities for high-throughput screening for catalytic function. Finally, the diversity of reactions catalyzed by simple peptide assemblies lends further support to the amyloid-first hypothesis of the emergence of enzymatic function.[15]

Acknowledgements

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