Enzyme Design

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Enantioselective Artificial Metalloenzymes Based on a Bovine Pancreatic Polypeptide Scaffold**

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The catalytic efficiency and high selectivities achieved by natural metalloenzymes are a continuing source of inspiration for the design of novel bioinspired catalysts. An emerging approach for creating artificial metalloenzymes involves the incorporation of a synthetic transition-metal catalyst into a protein binding site. By using covalent, dative, or noncovalent anchoring strategies, a variety of highly enantioselective metalloenzymes have been created.[1] To date, however, the choice of protein scaffold has been limited; only proteins such as avidin, streptavidin, bovine serum albumin (BSA), and apo-myoglobin, which have a sufficiently large pocket to bind the catalyst and still leave space for the substrates, have been applied successfully in enantioselective catalysis.[2] Yet, our work on DNA-based catalysis has demonstrated that biomolecular scaffolds that do not contain a pre-existing active site can, in principle, also supply the chiral environment for a catalyzed reaction.[3] Herein, we introduce a novel strategy for artificial metalloenzymes that involves grafting of a new active site onto a small natural peptide scaffold by introducing nonproteinogenic amino acids capable of binding a Cu^{2+} ion.[4,5] The resulting metalloenzymes catalyze Diels–Alder and Michael addition reactions in water with high enantioselectivities.

Bovine pancreatic polypeptide (bPP), a member of the pancreatic polypeptide family of peptide hormones,[6,7] was selected as the protein scaffold. bPP is 36 amino acids in length and adopts robust and closely packed tertiary and quaternary structures. It comprises a polyproline type II helix (residues 1–8), a turn (9–12), and an α helix (13–31), with the polyproline helix backfolded on the α helix (Figure 1a).[7c] Hydrophobic interactions drive dimerization in solution to give an antiparallel homodimer quaternary structure in solution (dissociation constant $K_d = 3.24 \times 10^{-7}$ M, pH 5.0).[7,8]

For synthetic convenience, the peptide was truncated to 31 residues (bPP1–31); residues 32–36 are disordered in the NMR structure and not involved in either tertiary- or quaternary-structure formation.[7c,8] Based on a calculated model for the dimer structure,[7a] Tyr7 was selected for replacement by a variety of amino acids containing a heteroaromatic side chain that are capable of binding a transition-metal ion, namely histidine (**bPP^{a}**) and 3- (**bPP^{c-e}**) and 4-pyridylalanine (**bPP^{b}**, Figure 1b and d). Residue 7 is located at the dimer interface and the distance to its counterpart on the other subunit would be in the right range to achieve bidentate coordination of a metal ion. As a result, the hydrophobic interface between the two subunits could potentially provide a chiral pocket for binding of reactive substrates.

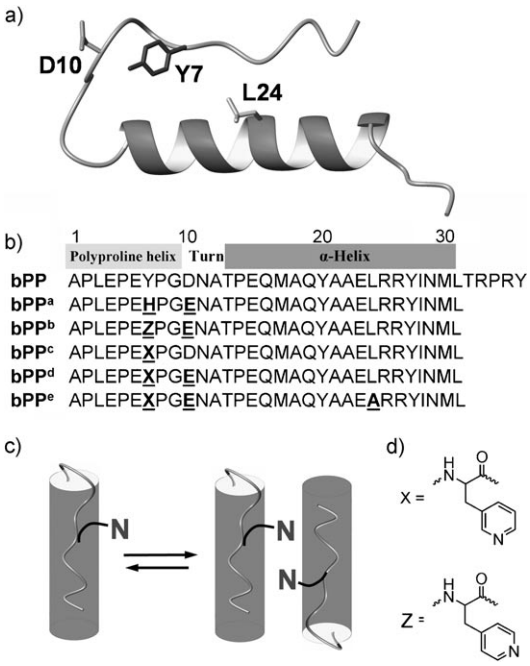

Figure 1. a) Molmol representation of monomeric **bPP** based on the NMR structure.[7c] Residues modified in the present study (Tyr7, Asp10, and Leu24) are labeled with the one-letter amino acid code. b) Amino acid sequence alignment of wild-type (wt) **bPP** and **bPP^{a-e}**, represented in the one-letter amino acid code. c) Schematic representation of the monomer/dimer equilibrium of **bPP^{x}**. d) Nonproteinogenic amino acids used: X: L-3-pyridylalanine; Z: L-4-pyridylalanine.

The bPP variants **bPP^{a-e}** were prepared by solid-phase peptide synthesis on a Rink amide resin by using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry, purified by preparative C18 reversed-phase (RP) HPLC, and characterized by electrospray and MALDI-TOF mass spectrometry. In all cases, analytical RP-HPLC of the purified peptides showed a single peak and the experimental and calculated molecular

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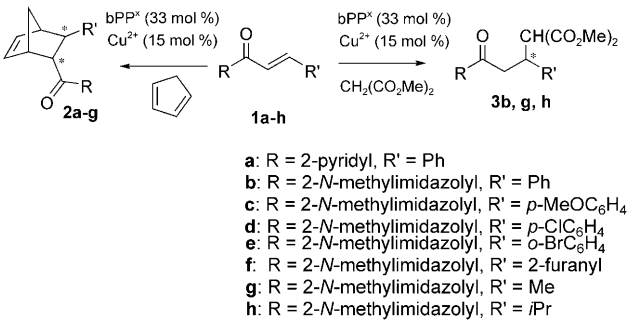
masses were in excellent agreement.[9] In the case of **bPP^{c}**, a small amount ($\approx 8\%$) of a side product was formed, which had a mass that was 18 amu lower than expected. The latter was attributed to aspartimide formation at position 10.[10] A D10E mutation was introduced in **bPP^{a,b,d,e}** to circumvent this problem. Indeed, the side product was not observed after the synthesis of **bPP^{a,b,d,e}**. An additional L24A mutation was included in **bPP^{e}** to increase the size of the pocket around the pyridyl-bound Cu^{II} ion.

The UV/Vis spectra of wt **bPP** and **bPP^{a}** are similar (λ_{max} value at 278 nm), whereas the spectra of **bPP^{b-e}** have a strong absorption between 259 and 268 nm, which is characteristic of pyridylalanine residues.[9] The circular dichroism spectra of **bPP^{a-e}** at room temperature are highly similar to that of wild-type bPP, with minima at (210 ± 2) nm and (222 ± 1) nm; these values are characteristic for a polypeptide with a significant degree of α-helix conformation. These data show that the modifications do not cause a significant disruption of the secondary structure.[8a] However, sedimentation-equilibrium analytical ultracentrifugation analysis at catalytically relevant concentrations indicates that **bPP^{d}** exists in a monomer/dimer equilibrium (association constant $K_a = 1.5 \times 10^2\,M^{-1}$; Figure 1c).[9] Truncated bPP (bPP1–31) has been reported to be dimeric,[8a] so the decrease in dimerization affinity has to be due to the Y7X mutation. In the presence of Cu^{II} ions, the dimerization affinity increases only slightly ($K_a = 3.9 \times 10^2\,M^{-1}$). A titration of $Cu(H_2O)_6(NO_3)_2$ into a 1 mM solution of **bPP^{d}**, monitored spectrophotometrically at 720 nm, showed approximately stoichiometric binding of Cu^{2+} ions to **bPP^{d}** (see Figure S3d in the Supporting Information), which indicates that the catalyst is predominantly monomeric. The binding of a Cu^{2+} ion to a single pyridine is relatively weak,[11] so this suggests that, in addition to the 3-pyridylalanine, other residues in the peptide are also involved in the binding of the Cu^{2+} ion. In view of the ultracentrifugation results, small but significant amounts of the copper-bound dimeric **bPP^{d}** are also likely to be present under catalytic conditions, that is, 0.2 mM **bPP^{x}** and 95 μM Cu^{2+} ions.

The catalytic potential of the peptides was evaluated by using the Diels–Alder reaction of azachalcone **1a** with cyclopentadiene as a model system (Scheme 1). The reactions were performed with 15 mol% of $Cu(H_2O)_6(NO_3)_2$ and 33 mol% of ligand **bPP^{x}** (2 ligands per metal center) in 3-(*N*-morpholine)propanesulfonic acid (MOPS) buffer solution (20 mM, pH 6.5). After reaction for 3 days at 5 °C, the Diels–Alder product **2a** was obtained as the only detectable product.

No conversion was found in reactions performed with wt bPP in the absence of copper.[9] With copper, the observed reaction was not enantioselective. Similarly, with **bPP^{a}** or **bPP^{b}**, no significant enantioselectivity was obtained (Table 1, entry 1). In contrast, **bPP^{c}** and **bPP^{d}**, gave the (−) enantiomer of **2a** in good yield and with *ee* values of 83 and 80%, respectively. The reaction proved to be ligand accelerated; the apparent second-order rate constants, k_{app}, with 0.1 mM $Cu(H_2O)_6(NO_3)_2$ in the absence and presence of **bPP^{d}** were (0.048 ± 0.0038) and $(0.17 \pm 0.013)\,M^{-1}s^{-1}$, respectively.[9] This corresponds to a 3.5-fold rate acceleration as a result of ligation of Cu^{2+} ions by **bPP^{d}**. Thus, the presence of any unbound Cu^{2+} ions will have only a minor effect on the *ee* value. Combined, these results demonstrate that a pyridine moiety is required and, furthermore, that the enantioselectivity critically depends on the structure of this group. Only in certain cases, as with the 3-pyridyl side chain, is an active site created that is capable of asymmetric induction.

A control experiment with the tripeptide EXP, that is, 3-pyridylalanine flanked by the same amino acids as in **bPP^{c-e}**, gave no enantioselectivity.[9] This demonstrates the importance of the second coordination sphere supplied by the peptide scaffold in asymmetric induction. A **bPP^{d}**/Cu^{2+} ion ratio of 2 or higher gave the best *ee* values; a ratio of less than 2 induced a progressive reduction in the *ee* value down to 49% for a ratio of 0.5 (see Figure S4 in the Supporting Information). A decrease in the copper loading to 5 mol%



Scheme 1. Asymmetric Diels–Alder and 1,4-addition reactions catalyzed by **bPP^{x}**–$Cu(NO_3)_2$ complexes in water.

Table 1: Results of Diels–Alder reactions catalyzed by Cu–**bPP^{x}** (Scheme 1).[a]

Entry	Substrate	**bPP^{a}** *ee* [%][b] (conv. [%])	**bPP^{b}** *ee* [%][b] (conv. [%])	**bPP^{c}** *ee* [%][b] (conv. [%])	**bPP^{d}** *ee* [%][b] (conv. [%])	**bPP^{e}** *ee* [%][b] (conv. [%])
1	**1a**	< 3 (96)	6 (65)[d]	83 (73)	80 (full)	4 (94)
2[c]	**1a**				74 (full)	
3	**1b**		< 3 (32)	65 (full)	14 (58)	< 3 (full)
4	**1c**			62 (3)	52 (9)	3 (7)
5	**1d**			39 (8)	23 (10)	4 (4)
6	**1e**			20 (3)	8 (< 3)	3 (21)
7	**1f**			13 (< 3)	8 (< 3)	9 (< 3)
8	**1g**			< 3 (full)	< 3 (96)	

[a] Typical conditions: 200 μM **bPP^{x}** and 95 μM $Cu(H_2O)_6(NO_3)_2$ in 20 mM MOPS buffer (pH 6.5) for 3 days at 5 °C, unless noted otherwise. The *ee* values are the average from 2 experiments and are reproducible within 2%. In all cases, the (−) enantiomer was found in excess. [b] For the *endo* isomer, *endo*/*exo* ≥ 95:5. [c] 5 mol% Cu^{2+} and 11 mol% **bPP^{d}**.

and in the **bPP^{d}** loading to 11 mol% still gave full conversion but with a slightly decreased *ee* value (Table 1, entry 2).

The scope of the reaction was investigated by using a variety of α,β-unsaturated 2-acyl imidazole substrates,[12] which are excellent substrates for Lewis acid catalysis in water (Scheme 1).[3c,d] When R′ = phenyl (**1b**; Table 1, entry 3), good conversion and enantioselectivity were obtained with Cu–**bPP^{c}**. Substitution of the phenyl ring with an electron-withdrawing or -donating group led to a dramatic reduction in the yield (Table 1, entries 4–6), although significant *ee* values were still obtained with **2c** and **2d**, that is, 62 and 39%, respectively. Replacement of the phenyl ring with a 2-furanyl group had a detrimental effect on both conversion and enantioselectivity (Table 1, entry 7). By contrast, excellent conversion to the Diels–Alder product was observed with R = methyl, albeit without enantioselectivity (Table 1, entry 8). Finally, with chalcone as the substrate, <3% conversion was observed, which suggests that bidentate binding of the substrate to the Cu^{2+} ion is required for activity, as was observed before.[2g,3d,9]

Cu–**bPP^{d}**, which has the D10E mutation, displays the same trends with the α,β-unsaturated 2-acyl imidazole substrates. This was expected because the D10E mutation is conservative and is not expected to significantly affect the structure of the peptide. A notable exception, however, is substrate **1b**, which gave rise to a significantly decreased *ee* value than that obtained with Cu–**bPP^{c}** (Table 1, entry 3). At present, the origin of this decrease is still not understood. **bPP^{e}**, which contains an additional L24A mutation, gave similar conversions but, surprisingly, displayed a complete loss of enantioselectivity; this result suggests a role for Leu24 in determining the enantioselectivity of the Diels–Alder reaction.

Encouraged by these results, we decided to explore the potential of these hybrid enzymes in the catalytic asymmetric Michael addition reaction in water (Scheme 1). The 1,4-addition reaction was performed by using dimethylmalonate as the nucleophile. In marked contrast to the Diels–Alder reaction, a modest conversion and no enantioselectivity were observed with substrate **1b** (Table 2, entry 1) with Cu–**bPP$^{c–e}$**. However, good conversion and high *ee* values were obtained with **1g**, up to 86% *ee* for product **3g** in the case of Cu–**bPP^{d}** (Table 2, entry 2). A decrease in the catalyst loading to 5 mol% led to slightly lower *ee* value of 75% (Table 2, entry 3). Interestingly, with **bPP^{e}**, which was nonselective in the Diels–Alder reaction, a good *ee* value was obtained.

Given the broad substrate scope of the analogous DNA-based catalytic reactions,[3] the high substrate selectivity of the bPP-based catalysts is notable. Substituents on the enone moiety of the substrate (R′) that are too large, such as a substituted phenyl group, lead to loss of activity and significant reduction of enantioselectivity. Activity is restored with smaller substituents, such as R′ = CH_3, but enantioselectivity is then only seen for the Michael addition. The enolate of dimethylmalonate, which is used as the Michael donor, is much larger than cyclopentadiene, which is used in the Diels–Alder reaction. Hence, a tentative conclusion is that the active site provided by the hybrid catalyst is compatible only with certain substrate/reactant combinations, such as **1a**/**1b** with cyclopentadiene or **1g** with the enolate of dimethylmalonate. Incompatibility with the structure of the active site leads to a loss of activity and/or enantioselectivity. In this sense, the present system resembles true enzymatic catalysts, which generally also have an active site optimized for one reaction. Current efforts are directed towards the elucidation of the oligomerization state and the active-site structure of the most efficient bPP-based artificial enzymes, which will provide more insight into the origin of the observed enantioselectivity.

In conclusion, we have developed a novel strategy towards the design of artificial metalloenzymes, which involves grafting of an active site onto an existing small natural protein scaffold by incorporation of a nonproteinogenic amino acid that is capable of binding a transition-metal ion. A key strength of the present approach is that an existing binding pocket in the protein is not required; this greatly expands the choice of protein scaffolds for artificial-metalloenzyme design. By using bPP-based catalysts, good enantioselectivities were obtained in the Cu^{2+}-catalyzed Diels–Alder and Michael addition reactions in water, that is, up to 83 and 86% *ee*, respectively. A particularly interesting feature of the present system is the high substrate selectivity, which is reminiscent of natural enzymes.

Table 2: Results of Michael addition reactions catalyzed by Cu–**bPP^{x}** (Scheme 1).[a]

Entry	Substrate	**bPP^{c}** *ee* [%][b] (conv. [%])	**bPP^{d}** *ee* [%][b] (conv. [%])	**bPP^{e}** *ee* [%][b] (conv. [%])
1	**1b**	<3 (25)	<3 (59)	<3 (15)
2	**1g**	66 (70)	86 (85)	65 (90)
3[c]	**1g**		75 (70)	
4	**1h**	<3 (10)	6 (54)	

[a] Typical conditions: 200 μM **bPP^{x}** and 95 μM $Cu(H_2O)_6(NO_3)_2$ (15 mol%; **bPP^{x}** to Cu^{II} ratio: 2.1) in 20 mM MOPS buffer (pH 6.5) for 3 days at 5°C, unless noted otherwise. [b] The *ee* values are the average of 2 experiments and are reproducible within 2%. [c] 5 mol% Cu^{2+} and 11 mol% **bPP^{d}**.

Experimental Section

Representative procedure for **bPP^{x}**–Cu^{2+}-catalyzed reactions: An aqueous solution of $Cu(H_2O)_6(NO_3)_2$ (24 μL, 1 mM) was added to **bPP^{d}** (200 μM, 250 μL) in 20 mM MOPS buffer (pH 6.5) at 0°C. A fresh stock solution (5 μL) of substrate in CH_3CN was added. After addition of freshly distilled cyclopentadiene (1 μL) or dimethylmalonate (3 μL) at 0°C, the reaction was mixed for 3 days by continuous inversion at 5°C. The product was isolated by extraction with Et_2O (2 × 1.5 mL). The organic phases were dried (Na_2SO_4) and evaporated under reduced pressure to give the product. The conversion and *ee* value were determined by RP-HPLC.

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