Published in final edited form as:

Angew Chem Int Ed Engl. 2008 ; 47(9): 1758–1761. doi:10.1002/anie.200704266.

Biomimetic Catalysis of Diketopiperazine and Dipeptide Synthesis

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Abstract

Entry for the Table of Contents Modular, supramolecular catalysts based on the coiled coil peptide scaffold and designed to mimic nonribosomal peptide synthetases are demonstrated to catalyze the formation of diketopiperazine and linear dipeptides for several aminoacyl substrates. We further demonstrate that the nature of the active site residues in the peptide catalysts can be used to effect directed intermodular aminoacyl transfer processes and govern the relative yields of diketopiperazine, linear dipeptide, and hydrolyzed substrate.

Keywords

biomimetic synthesis; catalysts; diketopiperazine; peptides; supramolecular chemistry

In recent years significant progress has been made in the design of synthetic peptide catalysts that carry out isolated chemical reactions similar to those catalyzed by enzymes, albeit with significantly lower efficiencies.[1,2] An unmet challenge in the *de novo* design of enzymes is to engineer peptides capable of bringing about more complex, multi-step synthetic processes. [3] One such biosynthetic pathway is that of diketopiperazine (DKP) formation, which minimally requires simultaneous binding and activation of two aminoacyl substrates, aminoacyl transfer to generate a linear dipeptide intermediate, and cyclization of the dipeptide to yield the product DKP.[4,5] Here we report the design and characterization of supramolecular peptide assemblies that catalyze DKP and dipeptide synthesis for a variety of aminoacyl substrates. The peptides covalently capture two aminoacyl substrates from solution, hold them in proximity to make the aminoacyl transfer step effectively intramolecular, and then release product in the form of DKP. We further establish that the nature of the active site residues in the short α -helical homo- or heterotetrameric peptide catalysts influences the relative yields of DKP, linear dipeptide, and hydrolyzed substrates, indicating that appropriate active site engineering might eventually be used to govern product elongation or termination via hydrolysis or cyclization.

The dedicated biosynthetic pathways employed to synthesize DKP sometimes involve nonribosomal peptide synthetases (NRPSs).[4] These modular multi-enzyme complexes catalyze a series of directed, intermodular aminoacyl transfer reactions between adjacent covalently-anchored aminoacyl thiolester substrates (Figure 1a).[6] Our designed catalysts[1] aim to functionally mimic NRPSs by relying on peptide self-assembly to juxtapose two cysteine residues, each used for the covalent capture of aminoacyl substrates from solution via transthiolesterification, at the helical interfaces of a coiled-coil[7] assembly (Figure 1b,c). The

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resulting high effective concentration[8] of aminoacyl donor and acceptor moieties, and potential electrostatic, pK_a , and/or general acid/base contributions provided by the flanking **X₁** and **X₂** residues, afford significantly enhanced rates of intermodular aminoacyl transfer. [1] The final required step in DKP synthesis, cyclization of the dipeptide intermediate, could similarly be accelerated by contributions from appropriate active site residues.

We anticipated that the most beneficial active site residues for DKP formation might differ from those identified in our earlier model studies[1] due to the additional mechanistic requirements of DKP synthesis. We therefore initially investigated stoichiometric aminoacyl transfer reactions involving pre-formed L-phenylalanine peptidyl thioesters of sequences **1–5**, which differ only in the active site **X₁** and **X₂** residues. Encouragingly, in all cases we observed by RP-HPLC aminoacyl transfer to form linear dipeptide intermediates bound to the coiled coil, followed by cyclization to yield DKP (Figure 2). The peptide active site residues significantly influenced the rates of both product formation and substrate hydrolysis (Figure 2, Figure S1). The observed concentration of free dipeptide produced was less than 3% in all cases, indicating that dipeptide cyclization is significantly more efficient than dipeptide hydrolysis. The highest yields of DKP were observed for sequences **1** and **2** containing His at the **X₁** position (Figure 2b), [1] likely because the imidazole group of the His side chain can provide general acid-base or proton transfer catalysis. Furthermore, for sequences **1** and **2** (**X₁** = His) we observe evidence of only the aminoacyl substrate loaded at Cys8 acting as the acyl-acceptor moiety (Figure 1b, *path a*) (no dipeptide species are observed bound at position 13), whereas for sequence **5** (**X₁** = Asp) we instead observe only evidence of the substrate anchored to Cys13 acting as the aminoacyl acceptor (Figure 1b, *path b*), suggesting that the **X₁** and **X₂** active site positions could be exploited to bring about directed aminoacyl transfer through appropriate active site engineering. At the **X₂** active site position, incorporation of an Asp residue appeared to stabilize the coiled coil-bound aminoacyl thioester substrates and dipeptide species relative to sequences with His or Ala at the **X₂** position (Figure 2c, Figure S1). Thus, simply changing the **X₂** active site residue from His (peptide **1**) to Asp (peptide **2**) significantly increases the relative concentration of linear coiled coil-bound dipeptide to DKP (Figure 2d). In a background reaction of the 3-mercaptopropionic acid thioester of L-Phe (5 mM), less than 1.0 μ M DKP was formed after 4 h under otherwise identical conditions.

We next examined the generality of the designed aminoacyl transfer process using homo- and heterotetrameric assemblies of sequence **2** preloaded with various aminoacyl thioester substrates (Table 1). In homotetrameric assemblies (entries 1–6), yields were highest for Phe, Met, and Leu. The supramolecular nature of the coiled-coil scaffold allowed us to mix equal amounts of two differentially pre-loaded derivatives of peptide **2** (entry 7), resulting in the formation of a 35 % yield of the mixed DKP product. While observation of the heteromeric product (Phe-Met DKP) supports the possibility of heterotetramer formation followed by aminoacyl transfer between the different anchored substrates, the procedure also generated homomeric DKP species (Phe-Phe and Met-Met), as would be expected considering that assembly of both the homo- and the heterotetrameric coiled coils results in productive complexes that juxtapose aminoacyl substrates. To circumvent such a formation of product mixtures, we next appropriately disabled one of the active site Cys residues in each peptide using the acetamidomethyl (Acm) protecting group, such that parallel homotetrameric assemblies are prevented from juxtaposing aminoacyl donor- and acceptor-moieties whereas heterotetrameric bundles form competent active sites (entries 8–9, Figure S2). Encouragingly, when sequences preloaded with Gly (entry 8) or His (entry 9) were mixed with a ~5-fold excess of a Phe-loaded peptide, we observed efficient aminoacyl transfer (81 % and 74 % total yields, respectively). In both reactions, no homo-DKP was found, supporting the proposed mechanism of intermodular aminoacyl transfer brought about by parallel heterotetrameric coiled coils.

Achieving turnover remains one of the most challenging aspects of biomimetic catalysis. We examined the potential for catalytic DKP formation in reaction cycles involving aminoacyl substrate loading from solution, intermodular aminoacyl transfer, and dipeptide cyclization to generate DKP while regenerating peptide catalyst **1** (Figure 3a). Using an L-Phe substrate (and a slightly lower pH value of 6.0 to reduce the rate of background DKP formation), significantly enhanced DKP formation was observed relative to a background reaction carried out in the absence of peptide **1**; furthermore, the amount of DKP produced was strongly dependent on the concentration of **1** present (Figure 3b). Derivatives of sequence **1** that were Ac-m-protected at either Cys8 or Cys13 effected almost no rate enhancement relative to the background reaction (Figure 3b), supporting the proposed intermodular mechanism of aminoacyl transfer between the Cys8 and Cys13 positions. We also examined the generality of catalytic DKP formation using several aminoacyl thiolester substrates (Figure 3c). However, only very modest turnover numbers were observed—with the L-Phe substrate approximately two equivalents of DKP were produced in 48 hours for all catalyst concentrations. One possible cause of low turnover in these reactions can be attributed to the formation of low (~25 %) steady-state levels of coiled coil-bound thiolesters. A juxtaposition of two loaded peptide species is required for DKP formation, but in a statistical association of peptides in which only 25 % are loaded, only 1/16 of helical interfaces would contain the requisite two anchored thiolesters. Combined with competing thiolester hydrolysis, this low level of productive interfaces could give rise to the poor product yields. The observed low turnover might therefore represent an inherent limitation of using randomly-assorting noncovalently-associated molecules as catalyst scaffolds, especially in cases where proximity is an important component of catalysis. Attempts to increase the steady-state concentration of loaded catalyst species by employing substrates with different thiol leaving groups or by sequestering the thiol released by substrate hydrolysis/transthiol esterification did not significantly improve catalyst turnover (data not shown). Another possible cause of low turnover is that a conformational requirement (such as an amide trans-to-cis isomerization of the coiled coil-bound dipeptide) limits DKP formation, although this seems unlikely considering the moderate-to-good DKP yields in the reactions initiated with pre-loaded peptides (Table 1).

The major remaining challenges in using simple coiled-coil assemblies to effectively mimic NRPSs will be in achieving higher turnover and control over product elongation and termination steps. It remains to be seen if these relatively simple peptides are capable of providing the subtle chemical effects required to synthesize longer, more complex peptide products.

Supplementary Material

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Figure 1. Schematic representations of aminoacyl loading and intermodular aminoacyl transfer in a) NRPSs and b) the designed coiled coil catalysts. c) Active site residues of aminoacyl transfer catalyst **1** modelled onto the crystal structure of a coiled-coil homotetramer.[9] The peptide sequences are shown on the right, with active site residues underlined.

Figure 2.

Product formation versus time for reactions initiated with prefomed bis-L-Phe thiolesters of sequences **1–6** (~100 μM peptide, 50 mM HEPES, pH 7.0, 10 mM tris-carboxyethyl phosphine [TCEP] as reducing agent, 50 μM acetamidobenzoic acid [Aba] as internal concentration standard). a) Reaction profile for peptide **2**, showing consumption of the bis-substrate loaded starting peptide ($\square$) and formation of coiled coil-bound linear dipeptide intermediate ($\diamond$), diketopiperazine (Δ), total aminoacyl transfer products (linear dipeptide intermediate plus DKP, $\circ$), and L-Phe (substrate hydrolysis, $\times$). b) DKP formation for sequences **1** ($\diamond$), **2** (+), **3** ($\square$), **4** (Δ), **5** ($\circ$), **6** ($\times$). c) Formation of total coiled coil-bound linear dipeptide intermediates for sequences **1** ($\diamond$), **2** (+), **3** ($\square$), **4** (Δ), and **5** ($\circ$). d) Comparison of DKP (shaded symbols) and coiled coil-bound linear dipeptide (open symbols) for sequences **1** (X_2 = His, triangles) and **2** (X_2 = Asp, circles).

Figure 3.

Catalytic DKP formation for reactions initiated with sequence **1** at various concentrations and free aminoacyl thiolester substrates (5 mM) in solutions containing 50 mM MES, pH 6.0, 10 mM TCEP, and 50 μM Aba. a) Reaction scheme depicting catalytic formation of DKP. b) DKP formation as a function of time for reactions initiated with the L-Phe mercaptopropionic acid thiolester substrate (5 mM) and peptide **1** at 78 μM (○), 50 μM (□), 25 μM (◇), 0 μM (×), or with the derivative of **1** Ac_m-protected at Cys8 (82 μM, △) or at Cys13 (86 μM, +). c) Background-subtracted DKP formation as a function of time for reactions initiated with peptide

1 (~100 μ M) and the 3-mercaptopropionic acid thiolesters (5 mM) of Met ($\circ$), Phe ($\square$), Leu (Δ), and Tyr ($\diamond$).

Table 1

Product yields for reactions involving peptide **2** preloaded with various aminoacyl thiolester substrates at Cys8 and/or Cys13.^[a]

Entry	Cys8 TE	Cys13 TE	[Pepl] (μM)	Time (h)	Products ^[b] (% Yield)
1	Phe	Phe	111	4	DKP (35), linear (31)
2	Met	Met	147	4	DKP (57)
3	Leu	Leu	99	4	DKP (43)
4	Tyr	Tyr	118	4	DKP (12)
5	Val	Val	175	4	DKP (<1)
6	D-Phe	D-Phe	125	4	DKP (20), linear (10)
7	Phe	Phe	64	4	Phe-Met DKP (35), Met-Met DKP (42), Phe-Phe DKP (8)
8	Met	Met	64	2	DKP (63), linear (18)
9	Acn	Acn	504	2	DKP (54), linear (20)
	Phe	Phe	316		

^[a]“Cys8 TE” and “Cys13 TE” refer to the aminoacyl thiolester loaded at the respective active site Cys residues, while “Acn” denotes the acetamidomethyl protecting group. For entries 7–9, two differentially-preloaded derivatives of peptide **2** were mixed to initiate the reaction.

^[b]“Linear” refers to the linear coiled coil-bound dipeptide species. Unless otherwise noted, the yield of this species was less than 10 %. For entry 7, the yield of Phe-Met DKP is based on the total concentration of peptide (128 μM), whereas the yields of homo-DKP species are based on the concentration of parent peptides (64 μM). For entries 7–9, yields are based on the concentration of the limiting substrate peptide.