Peptidic Catalysts

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Biomimetic Catalysis of Diketopiperazine and Dipeptide Syntheses

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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, multistep 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] Herein we report the design and characterization of supramolecular peptide assemblies that catalyze DKP and dipeptide syntheses for a variety of aminoacyl substrates. The peptides covalently capture two aminoacyl substrates from the solution, hold them in proximity to make the aminoacyl transfer step effectively intramolecular, and release product in the form of DKP. We also 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 by hydrolysis or cyclization.

The dedicated biosynthetic pathways employed to synthesize DKP sometimes involve nonribosomal peptide synthetases (NRPSs).[4] These modular multienzyme complexes catalyze a series of directed, intermodular aminoacyl transfer reactions between adjacent covalently anchored aminoacyl thiolester substrates (Figure 1 a).[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 by transthiolesterification, at the helical interfaces of a coiled-coil[7] assembly (Figure 1 b,c). The resulting high effective concentration[8] of aminoacyl donor and acceptor moieties, and possible electrostatic or general acid–base contributions provided by the flanking X^1 and X^2 residues, afford significantly enhanced rates for the intermodular aminoacyl transfer.[1] The final step required in DKP synthesis, cyclization of the dipeptide intermediate, could similarly be accelerated by contributions from appropriate active-site residues.

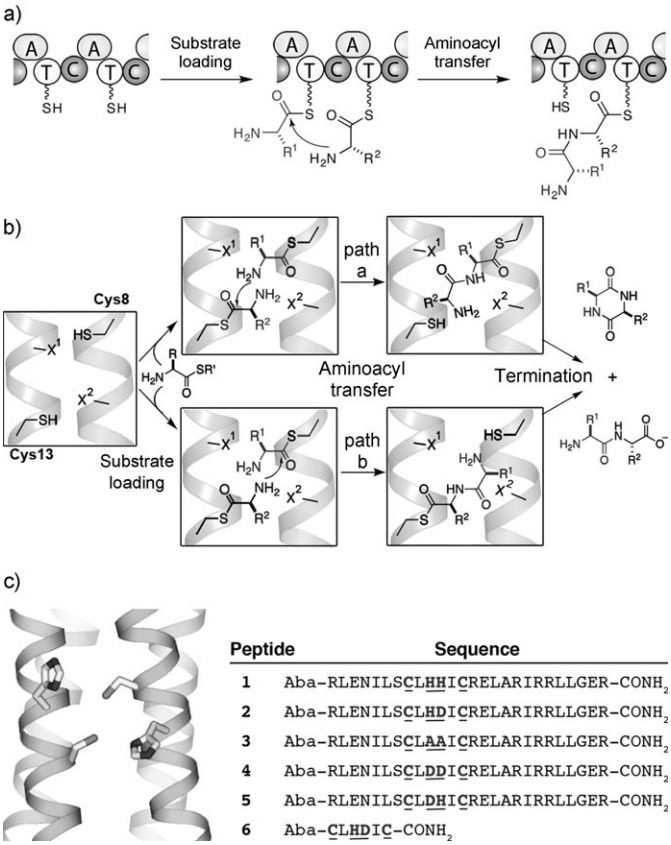

Figure 1. Schematic representations of aminoacyl loading and intermodular aminoacyl transfer in a) the nonribosomal peptide synthetases (NRPSs); b) the designed coiled-coil catalysts. X^1 and X^2 represent active-site residues that are modified. c) Active-site residues of aminoacyl transfer catalyst **1** modeled onto the crystal structure of a coiled-coil homotetramer.[9] The peptide sequences are shown on the right and the active-site residues are underlined.

We anticipated that the most beneficial active-site residues for DKP formation might differ from those identified in our earlier model studies[1] because of the additional mechanistic requirements of DKP synthesis. Therefore we initially investigated stoichiometric aminoacyl transfer reactions involving preformed L-phenylalanine peptidyl thiolesters of sequences **1**–**5**, which differ only in the active-site X^1 and X^2 residues. Encouragingly, in all cases we observed aminoacyl transfer to form linear dipeptide intermediates bound to the coiled coil and subsequent cyclization to yield DKP (RP-HPLC) (Figure 2). The peptide active-site residues significantly influenced the rates of both product formation and substrate hydrolysis (Figure 2, see the Supporting Information, 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

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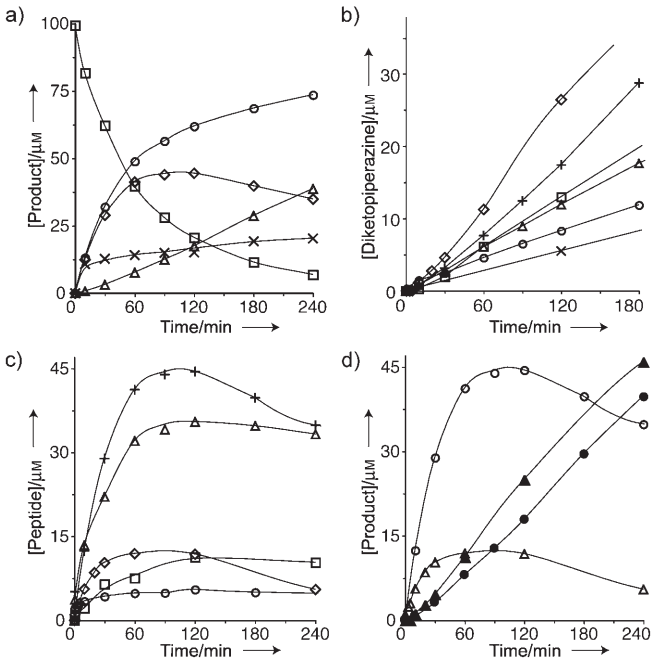
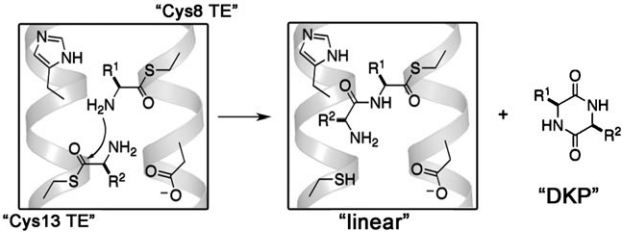


Figure 2. Product formation versus time for reactions initiated with preformed bis(L-Phe) thiolesters of sequences **1**–**6**. Reaction conditions: peptide (ca. 100 μM), 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0), tris-carboxyethyl phosphine (TCE; 10 mM) as a reducing agent, acetamidobenzoic acid (Aba; 50 μM) as an internal concentration standard. a) Reaction profile for peptide **2**, showing consumption of the bis-substrate-loaded starting peptide (□) and formation of coiled-coil-bound linear dipeptide intermediate (◇), diketopiperazine (△), total aminoacyl transfer products (linear dipeptide intermediate plus DKP, ○), and L-Phe (substrate hydrolysis, ×). b) DKP formation for sequences **1** (◇), **2** (+), **3** (□), **4** (△), **5** (○), **6** (×). c) Formation of total coiled-coil-bound linear dipeptide intermediates for sequences **1** (◇), **2** (+), **3** (□), **4** (△), and **5** (○). d) Comparison of DKP (solid symbols) and coiled-coil-bound linear dipeptide (open symbols) for sequences **1** (X^2 = His, triangles) and **2** (X^2 = Asp, circles).

dipeptide hydrolysis. The highest yields of DKP were observed for sequences **1** and **2** that contain His at the X^1 position (Figure 2b);[1] the high yields are 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** (X^1 = His) we observe that only the aminoacyl substrate loaded at Cys8 is acting as the acyl-acceptor moiety (Figure 1b, path a) (no dipeptide species are observed bound at position 13), whereas for sequence **5** (X^1 = Asp) we instead observe that the substrate anchored to Cys13 is acting as the aminoacyl acceptor (Figure 1b, path b). The results suggest that the X^1 and X^2 active-site positions could be exploited to bring about directed aminoacyl transfer through appropriate active-site engineering. At the X^2 active-site position, incorporation of an Asp residue appeared to stabilize the coiled-coil-bound aminoacyl thiolester substrates and dipeptide species relative to sequences with His or Ala at the X^2 position (Figure 2c, see the Supporting Information, Figure S1). Thus, changing the X^2 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 thiolester of L-Phe (5 mM), less than 1.0 μM DKP was formed after 4 hours 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 thiolester substrates (Table 1). In homotetrameric assemblies

Table 1: Product yields for reactions involving peptide **2** preloaded with various aminoacyl thiolester substrates at Cys8 or Cys13.[a]



Entry	Cys8 TE	Cys13 TE	[Pep] [μM]	*t* [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	64		Met-Met DKP (42),
					Phe-Phe DKP (8)
8	Acm	Gly	81	2	DKP (63), linear (18)
	Phe	Acm	504		
9	Acm	His	60	2	DKP (54), linear (20)
	Phe	Acm	316		

[a] Cys8 TE and Cys13 TE refer to the aminoacyl thiolester loaded at the respective active-site Cys residues and Acm 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.

(Table 1, entries 1–6), product 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 preloaded derivatives of peptide **2** (Table 1, entry 7), resulting in the formation of the mixed DKP product in a 35% yield. Whereas observation of the heteromeric product (Phe-Met DKP) supports the possibility of heterotetramer formation and subsequent aminoacyl transfer between the different anchored substrates, the reaction also generated the homomeric DKP species (Phe-Phe and Met-Met), which we expected because the assembly of both the homo- and the heterotetrameric coiled coils results in productive complexes that juxtapose aminoacyl substrates. To circumvent the formation of product mixtures, we disabled one of the active-site Cys residues in each peptide using an acetamido-

methyl (Acm) protecting group, such that parallel homotetrameric assemblies are prevented from juxtaposing aminoacyl donor and acceptor moieties, and heterotetrameric bundles form competent active sites (Table 1, entries 8 and 9, see the Supporting Information, Figure S2). Encouragingly, when sequences preloaded with Gly (Table 1, entry 8) or His (Table 1, entry 9) were mixed with an approximately fivefold 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). An L-Phe substrate was used at a slightly lower pH value of 6.0 to reduce the rate of background DKP formation. We observed significantly enhanced DKP formation 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** (Figure 3b). Derivatives of sequence **1** that were Acm-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 by using several aminoacyl thiolester substrates (Figure 3c). Only very modest turnover numbers were observed with the L-Phe substrate producing approximately two equivalents of DKP in 48 hours at all catalyst concentrations. One possible cause of low turnover in these reactions can be attributed to the formation of low (ca. 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 the helical interfaces would contain the requisite two anchored thiolesters. The low level of productive interfaces combined with competing thiolester hydrolysis, could give rise to the poor product yields. The low turnover observed might therefore represent an inherent limitation of using randomly assorting noncovalently associated molecules as catalyst scaffolds, especially when 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 or transthiolesterification, 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 preloaded peptides (Table 1).

The major challenges remaining for the use of simple coiled-coil assemblies to effectively mimic NRPSs are achieving higher turnover and better control of 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.

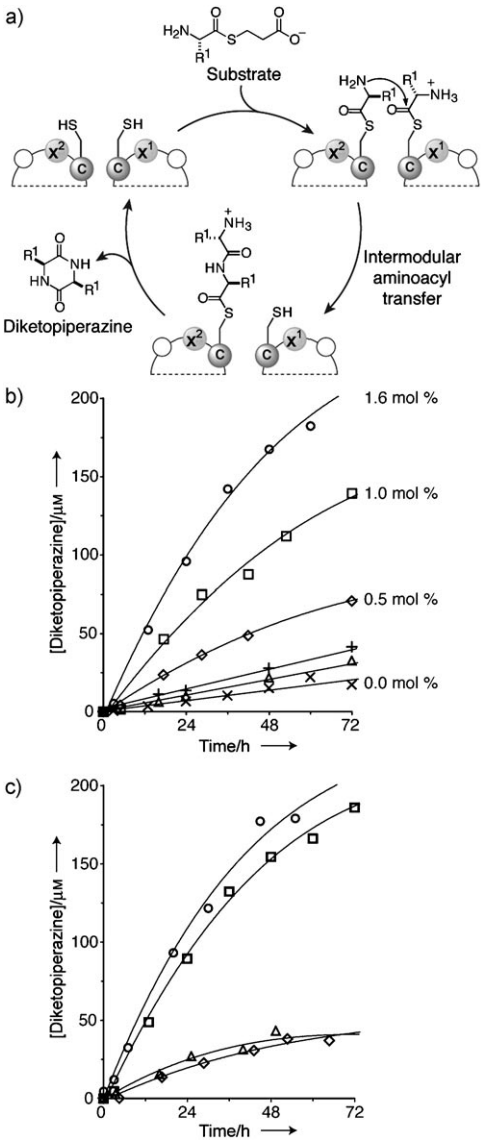


Figure 3. Catalytic DKP formation for reactions initiated with sequence **1** at various concentrations and free aminoacyl thiolester substrates (5 mM) in solutions containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.0), TCEP (10 mM), and Aba (50 μM). a) Reaction scheme depicting catalytic 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** Acm-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** (ca. 100 μM) and the 3-mercaptopropionic acid thiolesters (5 mM) of Met (○), Phe (□), Leu (△), and Tyr (◇).

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