

Phage display selection of peptides possessing aldolase activity

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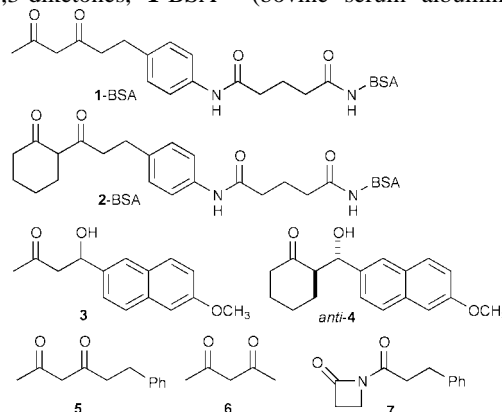
Received (in Corvallis, OR, USA) 10th January 2001, Accepted 1st March 2001
First published as an Advance Article on the web 3rd April 2001

Peptides catalyzing retro-aldol reactions were selected from a phage display peptide library using 1,3-diketones designed for the covalent selection of an enamine-based reaction mechanism.

The folded states of enzymes are key to their catalytic activity since structure can be used productively to modify the chemical reactivity of amino acid side chains, for example with electrostatic or hydrophobic effects, to build isolated reaction vessels, and to stabilize transition states of reactions. Small peptides are limited in their potential to catalyze reactions since they typically do not adopt well-defined structures. While the design of folded peptides has advanced tremendously in recent years, design at the level of catalysis is a challenging task. Rationally designed small catalytic peptides (14–42 amino acid residues peptides) have been reported that catalyze the decarboxylation of oxaloacetic acid,¹ ester hydrolysis,² and transesterifications.² Rational design, however, is limited in throughput.^{1b} The combination of rational design with library based methods may be an attractive approach to catalyst development. Phage display allows for libraries of greater than 10⁹ peptides to be probed for function, but this approach has not been used to select for catalytic peptides.³ Here we report the applicability of a mechanism-based covalent selection of catalytic peptides *in vitro*. Peptides possessing aldolase activity (aldolase peptides) that use an enamine-type mechanism were selected by reaction of peptide phage with 1,3-diketones that have been employed for the induction of aldolase antibodies⁴ (Scheme 1).

Amine catalyzed decarboxylation and aldol reactions share imine and enamine intermediates on their reaction coordinates. Accordingly, enzyme and antibody catalysts of these reactions are generally bifunctional catalysts capable of accelerating both decarboxylation and aldol reactions.⁵ In order to select aldolase peptides that use an imine–enamine reaction mechanism, we sought to exploit mechanistic homology by using as our starting point an 18-residue peptide (YLK-18-opt)^{1b} that catalyzes the decarboxylation of oxaloacetic acid. This peptide contains multiple lysyl residues that are key to its activity. To evolve the catalytic activity of this peptide, a 6-amino acid residue library was appended to the C-terminus of the peptide and the library was displayed on phage.⁶ The phage display peptide library YKLLKELLAKLKWLLRKLXXXXXX (X = any of the

natural 20 amino acids) was selected by three rounds of reaction with 1,3-diketones, 1-BSA^{4a} (bovine serum albumin) and

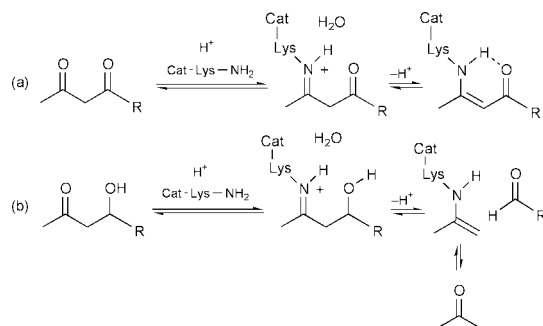


2-BSA.^{4c} Screening of selected phage by ELISA identified clones that bound to 1- and 2-BSA. The two most reactive peptides as judged by ELISA, FT-YLK-3 and FT-YLK-4, were chemically synthesized (C-terminal amide). Both peptides catalyzed the retro-aldol reactions of fluorogenic substrates (±)-**3**^{4b} and (±)-*anti*-**4**^{4c} in 5% CH₃CN–42.5 mM Na phosphate (pH 7.5). Since the solubility of the peptide FT-YLK-4 was low in buffer (around neutral pH) and prevented exact kinetic analyses, FT-YLK-3 was characterized in detail.

YLK-18-opt	YKLLKELLAKLKWLLRKL-NH ₂
FT-YLK-3	YKLLKELLAKLKWLLRKLGLPTCL-NH ₂
FT-YLK-4	YKLLKELLAKLKWLLRKLSDHCL-NH ₂
FT-YLK-3-R5	YRLLRELLARLRWLLRRLGLPTCL-NH ₂
FT-YLK-3-23S	YKLLKELLAKLKWLLRKLGLPTSL-NH ₂

The catalyzed reaction displayed saturation kinetics as described by the Michaelis–Menten equation. The parameters of FT-YLK-3 and of the template peptide YLK-18-opt are shown in Table 1.⁷ The *K_m* values were in mM range. The rate acceleration above the buffer catalyzed background reaction (*k_{cat}*/*k_{uncat}*) of the selected peptide FT-YLK-3 was 1400 and 500 for (±)-**3** and (±)-*anti*-**4**, respectively, and was superior to the template peptide YLK-18-opt. FT-YLK-3 demonstrated moderate enantioselectivity in the retro-aldol reaction of **3**. Studies performed using FT-YLK-3 (100 μM) and each of the enantiomers of **3** (1 mM) in 10% CH₃CN–40 mM Na phosphate (pH 7.5), revealed that the ratio of the initial velocities of FT-YLK-3-catalyzed reactions of (*R*)-**3**:(*S*)-**3** was 1.8:1.0.⁸

In order to probe the catalytic mechanism of the peptide, reactivity and mutational studies were performed. The catalyzed reaction was inhibited by the addition of diketone **5**. When FT-YLK-3 (100 μM) was mixed with diketone **5** (2 mM) at pH 7.5 for 2.5 h at rt and the retro-aldol activity assessed with (±)-**3** (1.2 mM) in 5% CH₃CN–42.5 mM Na phosphate (pH 7.5), the velocity of the retro-aldol reaction was < 15% that observed in the absence of **5**. Further, FT-YLK-3 formed a UV-observable enaminone with pentane-2,4-dione (**6**) when FT-YLK-3 (100 μM) was mixed with **6** (5 mM) as indicated by the appearance of a new absorption at 318 nm.⁴ The pH profile of the velocity of the enaminone formation indicates that FT-YLK-3 has a *pK_a*



Scheme 1 (a) Enaminone formation of peptide catalyst (Cat) with 1,3-diketone. (b) An enamine mechanism for the retro-aldol reaction.

Table 1 Kinetic parameters of the retro-aldol reactions^a

Peptide	(±)- 3			(±)-anti- 4		
	<i>K_m</i> , mM	<i>k_{cat}</i> , min ^{−1}	<i>k_{cat}</i> / <i>k_{uncat}</i>	<i>K_m</i> , mM	<i>k_{cat}</i> , min ^{−1}	<i>k_{cat}</i> / <i>k_{uncat}</i>
YLK-18-opt	1.8	2.1 × 10 ^{−4}	540	0.9	4.1 × 10 ^{−4}	170
FT-YLK-3	1.8	5.6 × 10 ^{−4}	1400	1.1	1.2 × 10 ^{−3}	500

^a Reaction conditions: [peptide] 100 μM, 5% CH₃CN–42.5 mM Na phosphate (pH 7.5) for (±)-**3** and, 5% DMSO–42.5 mM Na phosphate (pH 7.5) for (±)-anti-**4**, 25 °C. The first-order kinetic constant of the background reaction (*k_{uncat}*) was 3.9 × 10^{−7} for (±)-**3** and 2.4 × 10^{−6} min^{−1} for (±)-anti-**4**. The reaction was followed by monitoring the increase in fluorescence (λ_{ex} 330 nm, λ_{em} 452 nm).

lower than that typical of a lysine ε-amino group or a N-terminal amino group. The lowest p*K_a* is approximately 5.5. These results are consistent with peptide catalysis of the retro-aldol reaction using an enamine mechanism and chemically reactive amino group(s).

Since the low p*K_a* nucleophilic amino group of LysH93 of aldolase antibodies 38C2 and 33F12 can be specifically covalently modified with lactam **7**,⁹ FT-YLK-3 was treated with **7** to further study amino group reactivity. FT-YLK-3 (100 μM) was mixed with **7** (1 mM) and analyzed by MALDI-mass at 30 min, 2 h, and 6 h. Analysis indicated a time-dependent modification at multiple sites. At 30 min, 0–4 additions of **7** per molecule of FT-YLK-3 were observed while 1–5 and 1–7 modifications were observed at 2 and 6 h, respectively. In addition to lysine and a free amino terminus, FT-YLK-3 contains tyrosine, threonine, and cysteine residues that can react with **7**. Even with consideration of labeling at these 3 additional potential modification sites, the results suggest that more than one reactive amino group is labeled.¹⁰

To examine the effect of the lysyl residues, all five lysyl residues in FT-YLK-3 were replaced by arginine residues. Peptide FT-YLK-3-R5 displayed less than 5% of the catalytic activity of FT-YLK-3 with (±)-**3**. This result is also consistent with catalytic role for the ε-amino groups of the lysine residues of FT-YLK-3. In addition, 1 mM of lysine, arginine, tyrosine, proline, and mixtures of these amino acids, or lysyl-lysine did not catalyze the retro-aldol reactions described above as assayed in 5% CH₃CN–42 mM Na phosphate (pH 7.5).

Since no additional amino functionalities were selected in FT-YLK-3, improvement of the catalytic activity of the peptide may originate from structural changes to the peptide and/or the addition of factors such as acid–base catalysis and transition state stabilization. The CD spectra of FT-YLK-3 and YLK-18-opt (100 μM) in 45 mM Na phosphate buffer (pH 7.5) at 25 °C are shown in Fig. 1. For FT-YLK-3 the mean residue ellipticity at 208 and 222 nm were −3.04 × 10⁴ and −2.47 × 10⁴ deg cm² dmol^{−1}, respectively.¹¹ The CD spectra indicate that the peptide adopts an α-helical structure with an α-helical content of ~70%.¹² The template peptide YLK-18-opt showed a much reduced α-helical content under the same conditions, ~20%.¹³ These results suggest that selection of the 6 amino acid residue C-terminal extension in FT-YLK-3 stabilizes the α-helical conformation of the peptide. MALDI mass analysis of FT-YLK-3 revealed the peptide forms a covalent dimer though a monomer was the main species detected after storage in the buffer used for kinetic studies. The dimer of FT-YLK-3 formed

by a disulfide linkage did not preferentially form. The role of the Cys residue was assessed by synthesis of the Ser analog FT-YLK-3-23S. FT-YLK-3-23S catalyzed the retro-aldol reactions described above and the velocity of the catalyzed reaction of (±)-**3** (2 mM) was 75% that of FT-YLK-3. The ratio of the mean residue ellipticity of FT-YLK-3-23S at 208 and 222 nm were 0.8 and this ratio is the same as that observed in FT-YLK-3. The α-helix content of FT-YLK-3-23S was ~50%. The reduction in α-helical of FT-YLK-3-23S was correlated with a reduction in retro-aldolase activity. These results suggest that improved catalytic activity is intimately linked with stabilization of the conformation of the peptide.

In this study, we demonstrate that phage display and a compound designed to covalently trap catalysts that operate by a predefined mechanism can be used to directly select for small structured peptides that catalyze the aldol reaction. The coupling of phage display with this type of selection strategy provides a direct selection for peptide folding. For the aldolase peptide reported here, folding and catalytic activity were intimately linked.

This study was supported in part by the NIH (CA27489) and The Skaggs Institute for Chemical Biology.

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- The concentration of the peptide was determined by amino acid analysis.
- The purity of the enantiomeric substrates that were used in the reactions is as follows: (R)-**3**, 99.5% ee; (S)-**3**, 98% ee.
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- The order of the reactivity of the 5 lysyl residues of the peptide was not determined.
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- The α-helical content of YLK-18-opt is concentration dependent. See ref. 1b.

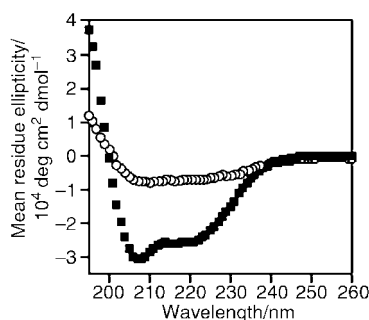


Fig. 1 CD spectra of peptides FT-YLK-3 (solid square) and YLK-18-opt (open circle). The CD spectra were recorded in 45 mM Na phosphate buffer (pH 7.5) at 25 °C at a peptide concentration of 100 μM.