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Modulating Pyridoxamine-Mediated Transamination Through a ββα Motif Peptide Scaffold

Michael A. Shogren-Knaak and Barbara Imperiali*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

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Abstract—A pyridoxamine coenzyme amino acid chimera (Pam) was incorporated into a designed ββα motif peptide to explore the ability of a small synthetic peptide scaffold to influence coenzyme mediated transamination. Structural characterization of this peptide by CD and NMR spectroscopy suggested that the pyridoxamine containing residue was accommodated into the sheet region of the motif without gross structural perturbations. To investigate the ability of the peptide architecture to influence the amount and distribution of transamination product in the conversion of pyruvic acid to alanine, a family of 18 related peptides, CBP01-CBP18, was rapidly synthesized and purified in parallel. These peptides were designed to generate different peptide environments for the pyridoxamine functionality within the context of the structured ββα peptide motif. Studies of peptide-mediated transamination revealed clear trends in stereospecific production of L-alanine as a function of substitutions at positions five and seven of the motif. Furthermore, new trends favoring the other enantiomeric product resulted from the addition of copper(II) ion, a known chelator of the transamination reaction intermediates. In the presence of copper(II) ion the amount of alanine product generated was increased by up to 32-fold relative to a pyridoxamine model compound in the presence of copper(II) ion. These functional results, accompanied by further CD and NMR spectroscopic analysis of CBP14, one of the CBP family of peptides, suggest that small synthetic ββα motif peptides can be used to influence the functional properties of coenzymes.

Introduction

Small synthetic peptides offer a powerful tool for exploring and influencing the reactivity of coenzymes—small, densely functionalized organic molecules that facilitate chemically difficult reactions in enzymes. Synthetic peptides combine many of the desirable features of proteins and small molecule model systems. Like proteins, these peptides can access complicated molecular architectures. The array of functional groups created by the peptide structure has the potential to influence the reactivity of the coenzyme in a manner similar to the structured active sites of enzymes. Additionally, like small molecule models, these systems are potentially simple enough to understand in atomic detail. Small peptide constructs also possess a number of practical advantages for experimental design: these systems can be assembled relatively easily using techniques of solid phase peptide synthesis (SPPS),[1] a wide range of targets is accessible because both natural and unnatural amino acid monomers can be employed, many related compounds can be explored using combinatorial techniques, and peptide constructs can be structurally characterized using standard biophysical methods.[2–4] To explore the potential of small synthetic peptides to influence the coenzyme reactivity, our efforts have focused on the chemically interesting process of transamination.

Transamination of α-keto acids to α-amino acids involves exchange of the amine functionality of an amino acid for the carbonyl of a keto acid to generate a new keto acid and amino acid (Scheme 1A). The uncatalyzed reaction is synthetically difficult, requiring harsh conditions and resulting in poor yields.[5] However, the coenzymes pyridoxamine-phosphate and pyridoxal-phosphate facilitate this process by acting as catalytic intermediaries in the reaction and achieve the same net reaction in a kinetically more accessible manner (Scheme 1B).[6] Mechanistically, the transamination half-reaction occurs in several steps, proceeding through ketimine, quinoid, and aldimine intermediates before generating the products (Scheme 2). Each of these mechanistic steps is made feasible through the assistance

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* Corresponding author. Current address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139; Tel.: 1-617-253-1838; e-mail: imper@mit.edu

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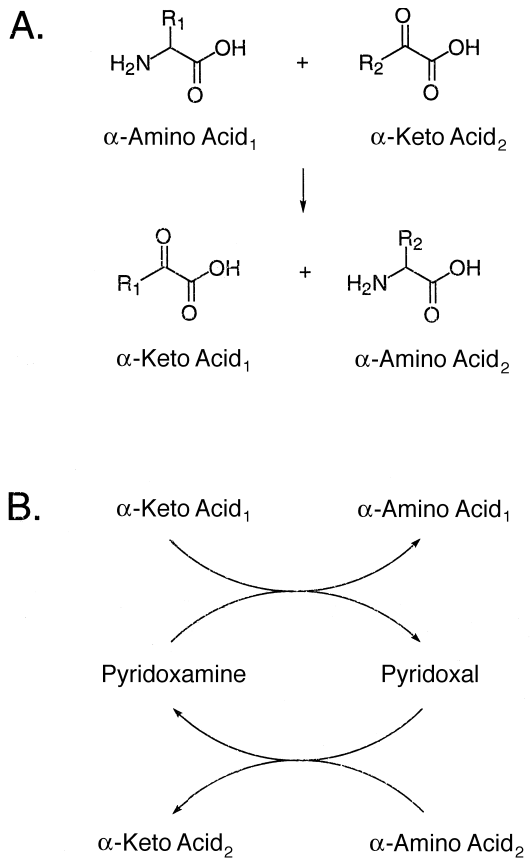


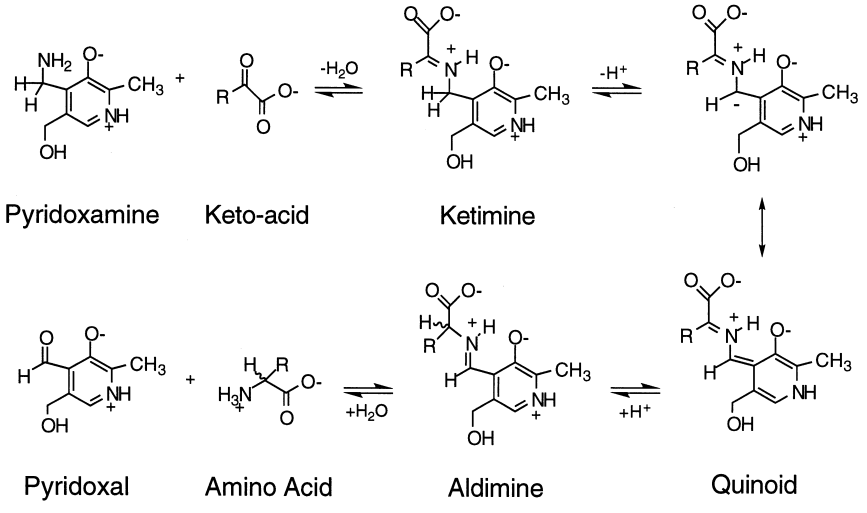
Scheme 1. (A) Net transamination reaction. (B) Cofactor-mediated transamination reaction.

of the coenzyme. In enzyme-catalyzed, pyridoxamine-dependent transamination the effectiveness of the coenzyme is enhanced and regulated by a well defined active site protein environment.[7]

Various model systems have been examined in an attempt to both emulate and illuminate the role of the enzyme environment. Small synthetic model systems,[8–12] as well as modified proteins,[13] have been designed to facilitate various mechanistic steps in the transamination process with some success. Previous work in our group has focused on incorporating either a pyridoxal coenzyme amino acid chimera (Pal) or pyridoxamine coenzyme amino acid chimera (Pam, Fig. 1) into synthetic peptide constructs, where the coenzyme functionality is covalently attached to the peptide main chain as part of an amino acid side chain. These peptide constructs have included short hairpin peptides[20,21] and semi-synthetic protein constructs.[14,15]

Recently, this laboratory has developed a series of small peptides that adopt a well defined supersecondary structure in the absence of disulfide bridges or metal chelation to serve as a scaffold for the development of functional peptides.[16–18] The BBA peptides include 23-residues assume a ββα supersecondary structure similar to a zinc-finger domain (Fig. 2). The β-hairpin region is stabilized by inclusion of a heterochiral type II′ turn element and by inclusion of residues with β-sheet propensities. The α-helix region is stabilized by residues with α-helical propensities and by an α-helix capping sequence. The packing of these two structural elements is promoted by hydrophobic interactions within the interior of the motif and hydrophilic residues on the exterior. With this structurally well-defined scaffold, the potential exists to create pyridoxamine containing peptides with augmented functional properties by exerting structural control over the peptide environment of the pyridoxamine cofactor.

To take advantage of this structural motif, Pam was incorporated into the BBA motif. Peptide BP1 (Fig. 2) incorporated Pam as a conservative mutation into a known structured ββα motif peptide, BBA4.[18] Spectroscopic analysis of BP1 revealed characteristics of the desired ββα-supersecondary structure. A family of peptides based on peptide BP1, CBP01-CBP18 (Fig. 2), was made to further influence the yield and optical induction of pyridoxamine mediated transamination. Functional and structural characterization of these constructs revealed peptides with augmented product generation and stereospecificity. These constructs represent the first success in making the BBA motif functional.



Scheme 2. Mechanism of the half-transamination reaction.

Results

Design of peptide BP1

The design of peptide BP1 (Fig. 2) was based on the sequence of ββα motif peptide, BBA4.[18] The Pam residue was placed into the β-sheet region of the peptide motif directly following the type II′ turn at position six. This substitution replaced a structurally related tyrosine residue and was not expected to perturb the supersecondary structure of the motif. To influence the function of Pam, a basic residue was also incorporated into the peptide sequence. In transaminase enzymes, a conserved lysine acts both as the general base in the rate limiting generation of quinoid intermediate[19–22] and as a general acid to reprotonate the quinoid species to afford a chiral amino acid product.[23,24] Furthermore, many of the other mechanistic steps in transamination can be acid or base catalyzed (Scheme 2). Molecular modeling indicated that replacing the serine at position five in BBA4 with an α,γ-diaminobutyric acid residue (Dab) might place the basic side chain of this residue to interact favorably with the coenzyme amino acid.

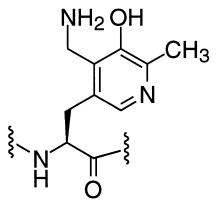
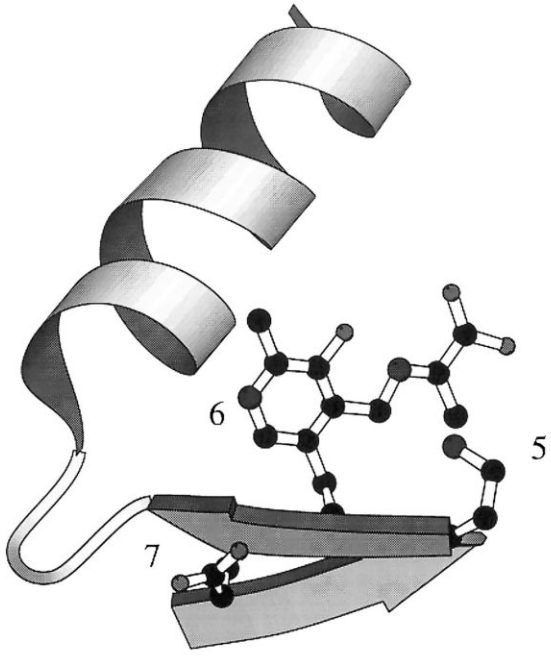


Figure 1. The pyridoxamine coenzyme amino acid chimera (Pam) incorporated into a peptide.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
BBA4	Ac	Tyr	Dap	Val	dPro	Ser	Tyr	Asp	Phe	Ser	Arg	Ser	Asp	Glu	Leu	Ala	Lys	Leu	Leu	Arg	Leu	His	Ala	Gly	NH_2
BP1	-	-	-	-	-	Dab	Pam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP01	Glc	-	-	-	-	His	Pam	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP02	Glc	-	-	-	-	Lys	Pam	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP03	Glc	-	-	-	-	Orn	Pam	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP04	Glc	-	-	-	-	Dab	Pam	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP05	Glc	-	-	-	-	Cys	Pam	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP06	Glc	-	-	-	-	Hcy	Pam	Asp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP07	Glc	-	-	-	-	His	Pam	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP08	Glc	-	-	-	-	Lys	Pam	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP09	Glc	-	-	-	-	Orn	Pam	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP10	Glc	-	-	-	-	Dab	Pam	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP11	Glc	-	-	-	-	Cys	Pam	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP12	Glc	-	-	-	-	Hcy	Pam	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP13	Glc	-	-	-	-	His	Pam	Dab	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP14	Glc	-	-	-	-	Lys	Pam	Dab	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP15	Glc	-	-	-	-	Orn	Pam	Dab	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP16	Glc	-	-	-	-	Dab	Pam	Dab	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP17	Glc	-	-	-	-	Cys	Pam	Dab	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CBP18	Glc	-	-	-	-	Hcy	Pam	Dab	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 2. Proposed structure of BP1/CBP04 based on the structure of peptide BBA4 with Pam depicted as the ketimine intermediate. Residues 5, 6, and 7 are shown to indicate substitution sites for peptides BP1 and CBP01-CBP18. Below are the sequences of peptides BBA4, BP1 and CBP01-CBP18. Non-standard amino acids include: α,β-diaminoproprionic acid (Dap); α,γ-diaminobutyric acid (Dab), D-proline (DPro), ornithine (Orn), homocysteine (Hcy), and the pyridoxamine amino acid (Pam). Peptides were either acetyl (Ac) or glycolyl (Glc) capped at the amino-terminus and were amide capped (NH_2) at the carboxy-terminus.

Structural characterization of peptide BP1

The secondary structural features of peptide BP1 were probed by circular dichroism (CD) spectroscopy (Fig. 3). Designed BBA peptides shown to adopt a ββα-secondary structure by 2-D NMR share several common CD spectral features at pH 4 and 8.[18] While the magnitude of the mean residual elipticity varies, the spectrum of each peptide shows an absolute minimum from 205 to 208 nM and a less intense minimum from 220 to 222 nM. The CD spectrum of BP1 exhibits similar characteristics with minima at 206 and 221 nM. At pH 4, the magnitude of the mean residual elipticity at 206 nM was identical to that of parent peptide BBA4, while the magnitude of the mean residual elipticity at 221 nM was about 16% greater. At pH 8, the pH at which transamination assays were performed, the CD spectra of BP1 and BBA4 were nearly identical. Thus, the CD spectra, particularly at pH 8, suggest that peptide BP1 shares some of the secondary structural characteristics of peptide BBA4.

To complement the CD analysis, two-dimensional (2-D) ^{1}H NMR was used to probe the supersecondary structure of peptide BP1. The solution structure of peptide BBA4 has been solved by 2-D NMR at pH 4.[18] A key feature of this structure is a set of packing interactions that are denoted by NOE crosspeaks between hydrophobic aromatic residues located exclusively on the sheet region and hydrophobic aliphatic residues found only on the helix. These unique NOE crosspeaks offer a strong diagnostic tool for the formation of supersecondary structure, being present only when the β-sheet region is packed against the β-helix domain. While full structural characterization of BBA4 has not been performed at pH 8, the pH at which transamination assays were performed, at this pH the peptide did exhibit aliphatic to aromatic NOEs crosspeaks expected in a well-defined hydrophobic core (data not shown). At pH 8, the spectra of peptide BP1 also showed aliphatic-to-aromatic side chain NOE contacts at intensities comparable to NOE crosspeaks for other residues (Fig. 4). The presence of these contacts suggests that the hydrophobic core also exists in peptide BP1.

Taken together, the structural analyses of peptide BP1 by CD and NMR spectroscopy suggest the overall ββα

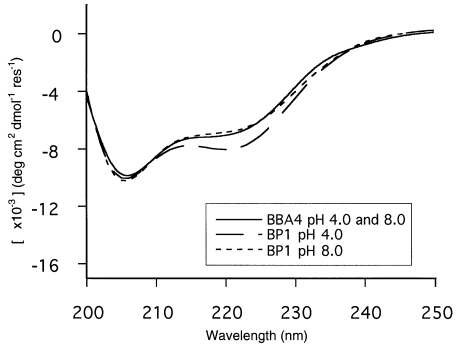


Figure 3. CD spectra of BBA4 and BP1 at pH 4 and 8.

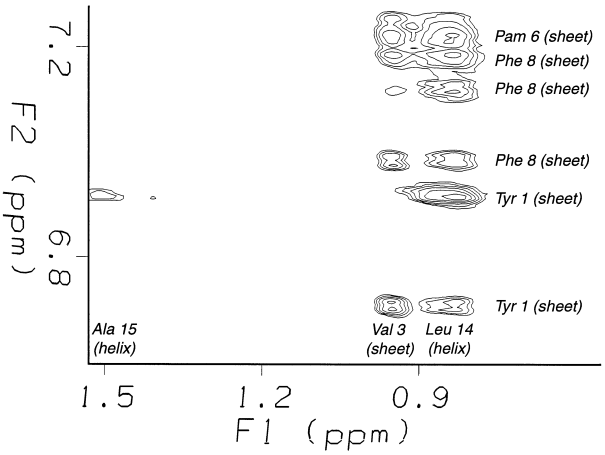


Figure 4. NOE side chain contacts for peptide BP1 in the aliphatic to aromatic region of the NMR spectrum at pH 8. Tentative NOE assignments based on BBA4. Chemical shifts are in ppm.

structure of peptide BBA4 is maintained at pH 8 with the incorporation of Pam and a basic residue under the conditions employed to assay transamination.

Functional characterization of peptide BP1

To assess the functional properties of peptide BP1, the peptide-mediated transamination reaction was studied using an HPLC/fluorescence assay.[25,26] In the transamination reaction an excess of pyruvic acid and D-phenylalanine were used and reactions were carried out in the presence of EDTA to prevent trace metal ion catalysis.[27] EDTA was added to both peptides and model compounds to both ensure that metal ion was not available to influence the reaction and to control for any intrinsic effects of the EDTA itself. The amount of each enantiomer of alanine produced from pyruvic acid was quantified at various time points by integrating the HPLC peaks resulting from injecting aliquots of the reaction mixture derivatized with an *ortho*-phthaldialdehyde/*N*-acetyl-L-cysteine solution. The initial rate of alanine production was determined from the time-dependent production of total alanine. The enantiomeric excess (% ee) for the reaction was determined from the relative quantities of each enantiomer present.

Relative to a 5′-deoxy-pyridoxamine model compound, dPam, peptide BP1 showed a slight improvement in properties with a 3.5-fold increase in the initial rate of alanine production and an optical induction of 15% ee in favor of the L-enantiomer. While peptide BP1 showed promise as a structural scaffold, improving the functional characteristics of the peptide required targeted perturbations of the coenzyme environment.

Design and synthesis of peptides CBP01-CBP18

Using BP1 as a template, a family of peptides, CBP01-CBP18, was designed to explore a range of coenzyme environments (Fig. 2). The replacement of serine 5 with Dab in BP1 indicated that similar substitutions might be tolerated at this position with a minimal loss of

structure. Six amino acids—histidine, lysine, ornithine, cysteine, homocysteine (Hcy), and Dab—were therefore placed at position five to investigate the ability of various basic residues to influence the course of transamination as either general acids or general bases. These amino acids provide basic side chains with a range of pK_as close to the pH of the transamination assays, along with a range of side chain lengths.

The placement of aspartic acid, glycine, and Dab, at position seven was also evaluated. This position flanks the C-terminal side of Pam on the exterior face of the β-sheet and the electrostatic, steric, and conformational differences of these residues was expected to perturb the relative geometry of the coenzyme and the basic residue at position 5. However, these substitutions were predicted to maintain the supersecondary structure of the motif, because non-hydrophobic residues had previously been shown to be tolerated at this site.[18] Together the peptides with substitutions at positions five and seven comprised the 18 peptides CBP01-CBP18.

The advantages of the small peptide scaffold approach allowed the above designs to be realized rapidly. An efficient technique for the parallel synthesis and purification of peptides CBP01-CBP18 has recently been developed.[28] The common carboxy-terminal residues 8–23 were assembled on resin by solid-phase peptide synthesis using an automated peptide synthesizer. The resin was then split into 18 smaller aliquots and the remaining residues were batch-coupled in parallel. Truncation peptides were observed following Pam coupling. To avoid time consuming HPLC purification, peptides CBP01-CBP18 were purified in parallel using a reversible biotin affinity tag purification method developed for this purpose. This technique rapidly afforded N-terminal glycolate-capped full-length peptides in 80% or greater purity and in quantities sufficient for a number of transamination assays.

Functional characterization of peptides CBP01-CBP18

For peptides CBP01-CBP18, peptide mediated transamination of an α-keto acid to the corresponding α-amino acid was probed as described for peptide BP1, except that initial amino acid production (less than 10% of a turnover) and optical induction information were determined from duplicate analyses of a single timepoint (Table 1).

Peptides CBP01-CBP18 demonstrated a range of functional behavior in the metal ion-free transamination of pyruvic acid to alanine. Relative to dPam assayed under identical conditions, all peptides showed at least a threefold increase in the amount of alanine produced in 20 h, and peptide CBP13 exhibited a 5.6-fold increase. For all peptides a slight optical induction favoring L-alanine was observed, while no significant optical induction was observed for dPam under the same assay conditions. The level of optical induction varied from a slight excess of L-alanine to 27% ee for peptide CBP14.

Interesting trends in stereoselectivity were seen for alanine production with peptides CBP01-CBP18. In the absence of metal ions, changing either residue at position five or seven produced independent trends (Fig. 5(a)). At position five, lysine gave the greatest optical induction, and in general longer side chain lengths resulted in greater optical inductions. At position seven, aspartic acid and glycine afforded similar degrees of optical induction, while inclusion of Dab produced enhanced optical induction.

In the presence of metal ions, coenzyme-substrate chelates are known to form,[27] and different functional properties were observed. The presence of copper(II) ion resulted in augmented production of alanine for peptides CBP01-CBP18 relative to the dPam model compound in the presence of copper(II) ion. All peptides

Table 1. Sequence substitutions at positions five and seven for peptides CBP01-CBP18 relative to peptide BP1. Comparison of initial product formation and enantiomeric induction with pyruvate

			Pyv, D-Phe, EDTA		Pyv, D-Phe, Cu(II)	
Peptide	Position 5	Position 7	Ala/dPam	% ee L-Ala	Ala/dPam	% ee D-Ala
CBP01	His	Asp	3.4	10	24.4	8
CBP02	Lys	Asp	3.5	14	23.9	15
CBP03	Orn	Asp	4.4	10	25.4	19
CBP04	Dab	Asp	3.5	8	21.3	20
CBP05	Cys	Asp	3.0	4	23.3	10
CBP06	Hcy	Asp	3.0	10	24.6	7
CBP07	His	Gly	3.7	11	23.9	22
CBP08	Lys	Gly	3.8	18	23.4	27
CBP09	Orn	Gly	4.1	12	25.8	28
CBP10	Dab	Gly	4.2	2	26.2	37
CBP11	Cys	Gly	3.9	7	27.3	31
CBP12	Hcy	Gly	3.0	16	21.7	21
CBP13	His	Dab	5.6	24	31.7	3
CBP14	Lys	Dab	4.7	27	24.4	5
CBP15	Orn	Dab	3.8	23	20.1	7
CBP16	Dab	Dab	4.8	16	26.4	14
CBP17	Cys	Dab	4.8	8	25.1	13
CBP18	Hcy	Dab	4.1	17	22.1	6

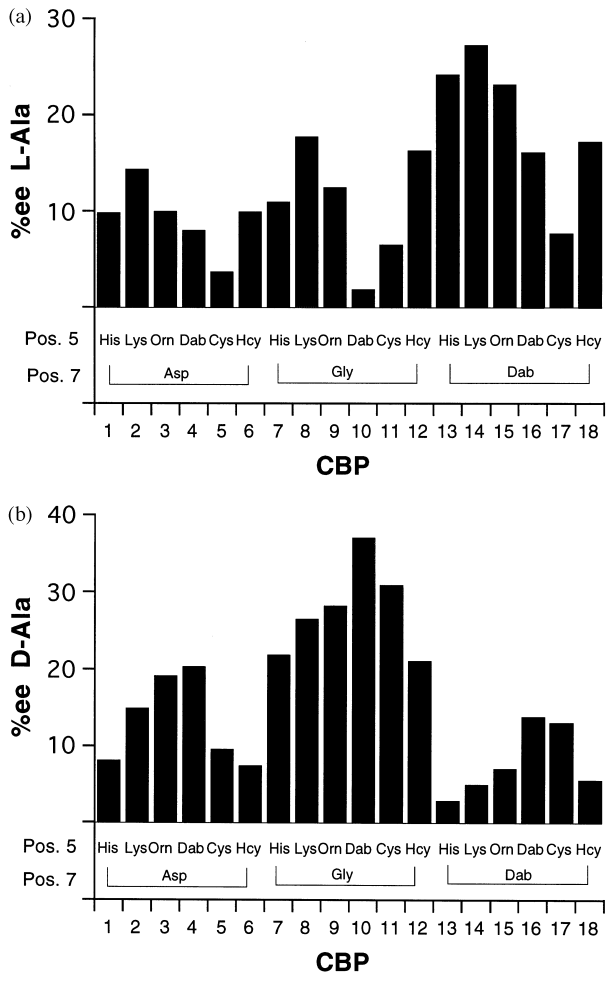


Figure 5. Observed optical induction in the production of alanine (a) in the absence of metal ion and (b) in the presence of copper(II) ion for peptides CBP01-CBP18. Peptide substitutions at position five and seven are indicated.

showed at least a 20-fold increase in product yield relative to dPam, and peptide CBP13 demonstrated a 31.7-fold increase in alanine production.

Additionally, the introduction of copper(II) ions resulted in dramatic and different stereochemical trends (Fig. 5(b)). In contrast to the metal ion-free conditions, D-alanine production was favored in the presence of a stoichiometric amount of divalent copper ion. The degree of optical induction ranged from less than 5% up to 37% enantiomeric excess of D-alanine for peptide CBP10. Furthermore, for the basic residues at position five, the greatest optical induction was produced by Dab, and in general shorter side chain lengths favored larger optical inductions. Peptides with glycine at position seven, resulted in the highest stereoselectivity, while those with Dab resulted in the lowest stereoselectivity.

Structural characterization of peptide CBP14

The trends in optical induction observed with peptides CBP01-CBP18 suggest that the pyridoxamine functionality might exist within a defined peptide environment. To support this proposal, the structural features of peptide CBP14, the CBP peptide with the greatest optical induction in the absence of copper(II) ion, were investigated by CD and 2-D NMR spectroscopy in a manner similar to peptide BP1.

CD spectral analysis of CBP14 revealed spectral properties similar to peptide BP1 and previously studied BBA peptides (Fig. 6). At both pH 4 and 8, peptide CBP14 exhibited an absolute minimum of mean residual elipticity at 205 nM with an equal magnitude. Additionally, at both pH 4 and 8 a minimum was observed at 222 nM, with a 10% greater absolute mean residual elipticity at pH 4 relative to pH 8. While the absolute mean residual elipticities at 205 and 222 nM were greater than those observed for either BP1 or BBA4, other BBA peptides shown to have ββα-structure have varied in the magnitude of the observed mean residual elipticities.[18] Thus, the CD spectral data suggest that peptide CBP14 shares secondary structural features with peptides BP1 and BBA4.

The 2-D NMR spectrum of CBP14 at pH 8 also suggests that peptide CBP14 adopts the supersecondary structure of the ββα motif peptides (Fig. 7). Like BBA4 and BP1, the aliphatic to aromatic portion of the NOESY spectrum of peptide CBP14 shows NOE crosspeaks between aliphatic and aromatic side chains. Because these crosspeaks are only expected for a species in which the sheet and helix domain are packed, the presence of these crosspeaks suggests that CBP14 adopts the ββα motif supersecondary structure.

Discussion

The BBA peptide as a structural scaffold

Structural characterization of peptide BP1 by CD and NMR spectroscopy indicated that the peptide exhibits hallmark features of ββα motif secondary and supersecondary structure. Thus, it appears that a BBA motif can accommodate Pam and a basic residue while maintaining the desired ββα motif structure that has the

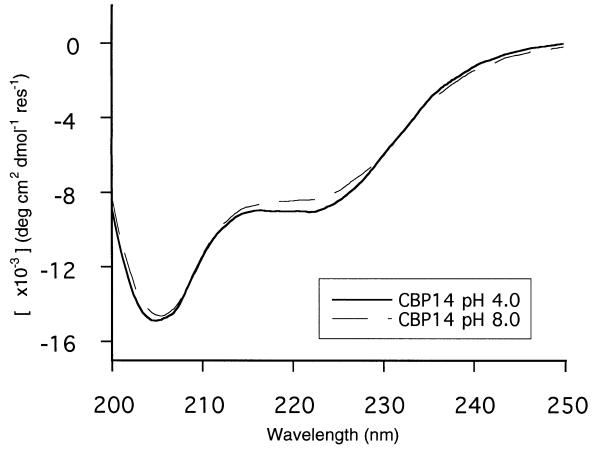


Figure 6. CD spectra of CBP14 at pH 4 and 8.

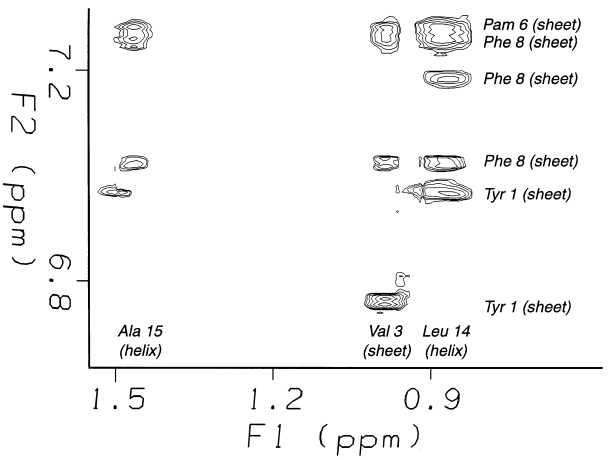


Figure 7. NOE side chain contacts for peptide CBP14 in the aliphatic to aromatic region of the NMR spectrum at pH 8. Tentative NOE assignments based on BBA4. Chemical shifts are in ppm.

potential to support a defined coenzyme environment. That such substitutions would be tolerated within a small designed ββα motif peptide such as peptide BBA4 was not initially obvious: incorporation of the Pam residue within the helical region of the motif (His17 to Pam) resulted in a peptide with a CD spectrum that was sensitive to changes in pH, while introduction of Pam into the hydrophobic core of peptide BBA4 (Phe8 to Pam) resulted in a peptide with a pH-independent CD spectra, but lacked NOEs characteristic of supersecondary packing (data not shown).

Based on the structural characteristics of prototype peptide BP1, on the clear functional trends in optical induction for the transamination of pyruvate, and on the CD and NMR analysis of peptide CBP14, it appears that peptides CBP01-CBP18 may also possess the gross supersecondary structure of the ββα motif while possessing a coenzyme environment that influences transamination. At this point the full structural features of the Pam containing BBA peptides has not been determined.

The BBA peptide as a functional scaffold

The BBA peptide motif has proven to be a rugged platform for influencing pyridoxamine mediated transamination. Because of the synthetic and modular nature of the BBA peptides, these constructs provide rapid access to a range of coenzyme environments.

The BBA peptide motif has the capacity to augment the production of amino acid above the coenzyme alone. In the presence of copper(II) ion, peptides CBP01-CBP18 demonstrate increases in the amount of alanine produced during the assay period relative to the dPam control under the same conditions by at least 20-fold, with peptide CBP13 showing a 31.7-fold increase. Previous studies under similar copper(II) ion conditions with a Pam containing peptide designed to be unstructured (Ac-Thr-3′Pyridylalanine-Gly-Gly-Pam-Gly-NH_2) showed an increase in initial rate of alanine production of only 1.8-fold (unpublished results). Thus, the observed increase in amino acid production appears not to result simply from incorporating the coenzyme functionality into a peptide backbone and may instead be due to the environment created by the ββα motif. The extent to which the full BBA peptide structure is required for the observed functional effects has not been determined.

The BBA peptide motif also has the ability to influence the stereochemical outcome of the transamination reaction. In the absence of metal ions, L-alanine is the favored transamination product, while in the presence of copper(II), the opposite enantiomer is favored. Copper(II) ion is known to be coordinated by the transamination intermediates and has the potential to accept other ligands. The difference in influencing the stereochemical outcome of transamination suggests that the BBA peptide motif has the capacity to interact differently with the metalated and unmetalated form of the coenzyme functionality. Furthermore, this optical induction is modulated by substitutions in the peptide sequence. Both in the presence and absence of divalent copper sequence changes at position seven resulted in different levels of optical induction independent of the nature of the basic residue at position five. Moreover, the identity of the basic residue at position five resulted in discernible trends. In the absence of metal ions, longer side chain residues resulted in increased production of L-alanine, with lysine producing the greatest degree of optical induction. In the presence of copper(II) ion, this trend is reversed, with basic residues with shorter side chains resulting in increased production of D-alanine, with the Dap residue producing the greatest increase. Modeling of the CBP peptides shows that the side chains of the basic residue at position five can interact with the pyridoxamine functionality. While the extent to which the full BBA peptide structure is required for the observed functional effects has not yet been determined, the observed trends do indicate that changes in the coenzyme environment can result in measurable functional differences between peptides.

Conclusion

Peptides BP1 and CBP01-CBP18 constitute the first use of a ββα motif peptide to generate functional peptides and this study demonstrates the potential utility of the scaffold. Additionally, these results highlight the flexibility of using small peptide constructs to influencing coenzyme-dependent catalysis. Structural characterization of peptides BP1 and CBP14, in addition to functional characterization of peptides CBP01-CBP18, suggest that amino acid residues can be incorporated into a BBA peptide with conservation of the overall BBA peptide structure. Furthermore, at least some elements of the BBA structure appear to provide a unique environment for the coenzyme to generate a range of functional properties within the structured motif. Further structural and functional characterization of these peptides will clarify the source of these properties. This information, in conjunction with the techniques made possible by the small peptide approach, should allow for

the continued exploration and improvement of small systems that mediate rapid and selective transamination.

Experimental

Materials

N^{α}-9-Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids and amino acid reagents were purchased from Millipore or PerSeptive Biosystems. The Pam residue was synthesized as previously described.[29] PAL-PEG-PS resin was purchased from PerSeptive Biosystems. The Vac-man vacuum system was purchased from Promega and the disposable polypropylene columns and valves were obtained from BioRad.

Peptide modeling and synthesis

Peptide design and modeling was performed using Insight II software (Biosym Technologies, San Diego, CA, USA).

Peptide BP1, the parent batch of resin for peptides CBP01-CBP18 (residues 8–23), and peptide CBP14 were made by solid-phase peptide synthesis performed on a Milligen 9050 automated peptide synthesizer. Peptides were synthesized on a 200 μmol scale from PAL-PEG-PS resin (1 g, 0.2 mmol/g). Amino acids were double coupled in fourfold excess, except for Pam, which was double coupled at 2.5-fold and 1.5-fold excess. Peptide coupling was performed with HOBt/HBTU chemistry, with the exception of Pam, which was coupled by HOAt/HATU chemistry. The Fmoc-amines were deprotected using a 20% piperidine/DMF wash. Between each new residue coupling, free amines were capped with a 0.3 M acetic anhydride/HOBt solution in 9/1 DMF/DCM.

Residues 1–7 of peptides CBP01-CBP18 were synthesized in parallel using batch solid-phase peptide synthesis techniques. The parent batch of resin was divided in 18×50 mg (6.4 μmol theoretical) portions. All residues were double coupled with 4 equiv amino acid (0.256 M), 4 equiv HOBt (0.256 M), 4 equiv HBTU (0.256 M) and 9 equiv diisopropylethylamine (0.576 M) in 89 μL DMF (Pam was coupled with a 2.5/1.5-fold excess with HOAt/HATU chemistry) for 2 h. Coupling of the N-terminal reversible biotin affinity tag was accomplished under conditions described elsewhere.[28] The extent of residue coupling was tested by Kaiser test on one of the 18 peptides after each coupling round. Acetylation steps were performed for 30 min using the acetylation solution described earlier. Deblocking steps were performed for 30 min with a 20% piperidine/DMF solution. The resin was washed with 20 mL of DMF between reaction steps and was occasionally agitated manually during the coupling step.

Peptides BP1, CBP01-CBP18, and CBP14 were deacetylated prior to acid cleavage to remove an acetyl group from the phenolic oxygen of the Pam residue.[29] Applying the previously described deacetylation conditions[29] to peptide BP1 resulted in isomeric products. Subjecting the peptide resin to 20% piperidine in DMF for 12 h resulted in full deacetylation without any isomeric products. Resin was subsequently washed thoroughly with DMF and DCM and lyophilized to dryness. Peptide was cleaved from the resin with an excess of reagent K (trifluoroacetic acid/phenol/H_2O/thioanisole/ethanedithiol, 82.5/5/5/5/2.5) for 2 h to afford the deprotected peptide.[30] Excess TFA was removed with nitrogen gas and was triturated with 2/1 ether/hexane. The resulting white pellet was resuspended in water and further purified by reverse-phase HPLC. Peptides CBP01-CBP18 were rapidly purified in parallel using an avidin affinity column method described elsewhere.[28]

Peptide quantification

Purified peptide stocks were quantified by UV–vis spectroscopy under denaturing conditions using a Beckman DU 7500 Spectrophotometer in a 1 cm path length quartz cuvette. Typically, peptide stock was mixed with an equal volume of a 4.8 M guanidine hydrochloride solution buffered at pH 7.5 with 0.2 M HEPPS. The concentration of the original stock was calculated from the absorbance at 322 nM with and ε_{322} of 5610 $M^{-1}\,cm^{-1}$, where ε was initially determined from a Pam containing peptide quantified by quantitative amino acid analysis.

Circular dichroism studies

CD experiments were performed on a Jasco J-600 spectropolarimeter or an Aviv 62DS Circular Dichroism Spectrometer. CD samples were prepared by dilution of peptide stocks to concentrations between 50 and 200 μM in degassed water for experiments performed at pH 4 or in 0.01 M pH 8 Tris buffer for experiments performed at pH 8. The pH of the sample was adjusted to either 4 or 8 by addition of 0.01 N NaOH. CD measurements were performed at room temperature in a 0.1 cm path length quartz cell with the optical chamber continually flushed with dry N_2 gas. Scans were collected in the range 195–250 nM, with a band width of 1.5–2.0 nM, a sensitivity of 50 mdeg, a scan speed of 50 nM/min, a time constant of 0.5 s, and a scan resolution of 0.2 nM. Four accumulations per sample were performed. CD spectra were analyzed in KaleidaGraph software, version 2.11 and are reported in (deg×cm^2)/(dmol×amide bonds).

NMR studies

2-D NMR experiments were performed on a 500 MHz Bruker Instrument AMX500 spectrometer or a Varian 600 MHz Unity Plus instrument. NMR samples were prepared in either a 90/10 H_2O/D_2O solution or a 99.98% D_2O solution with dioxane or dimethyl sulfoxide as an internal standard. Peptide concentration ranged from 2–10 mM. The pH was adjusted to 8 with small additions of 0.1 N NaOH or 0.1 N NaOD. Nuclear Overhauser effects (NOEs) were detected using spin-locked rotating-frame nuclear Overhauser effect spectroscopy (ROESY) with a 400 ms mixing time,[31] or nuclear Overhauser effect spectroscopy (NOESY) with a

250 ms mixing time.[32] Water suppression was obtained using presaturation during the relaxation delay. Spectra were processed and analyzed in Felix 95 (Biosym Technologies, San Diego, CA, USA) and NOESY spectra were baseline corrected.

Transamination assays

For peptide BP1, a typical assay was performed as follows: to a small Eppendorf was added 2 μL of a 10 mM EDTA solution (100 μM), a 10 μL of a 2 mM BP1 stock (100 μM), and 148 μL of a pH 8, 0.1 M HEPPS buffer with a μ of 0.29, 0.02% NaN_3. The solution was equilibrated at room temperature for 10 min, and the reaction was initiated by the addition of 40 μL of a fresh 50 mM sodium pyruvic acid/50 mM D-phenylalanine solution (10 mM of each). Assays proceeded at room temperature and over 24 h a number of 10 μL time-points were collected. For peptides CBP01-CBP18 the total volume, temperature, and order of addition were the same. However, EDTA or $CuCl_2$ and peptide were added to a final concentration of 10 μM. Reactions were initiated with 40 μL of a 50 mM sodium pyruvic acid/50 mM D-phenylalanine solution being added (10 mM of each) and were stopped before reaching 10% of a single turnover. Reactions with copper(II) ion were quenched after 1 h with a fivefold excess EDTA. Metal free assays were stopped after 20 h. All samples not derivatized immediately were flash frozen and stored at −80°C. Transamination assays for the dPam model compound were performed both in the presence and absence of copper(II) ion under the conditions described for peptides BP1 and CBP01-CBP18.

The amino acids generated in the assay were derivatized with saturated sodium borate buffer and freshly prepared *N*-acetyl cysteine (5 mg/mL)/OPA incomplete solution from Sigma according to literature procedure.[25,26] To derivatize samples from BP1, 20 μL of the saturated sodium borate buffer was added followed by 40 μL of the OPA solution. The reaction mixture was vortexed and then allowed to sit for exactly 5 min at room temperature, before one half of the reaction mixture was injected onto the HPLC (36 μL). For samples from peptides CBP01-CBP18, 100 μL of sodium borate buffer and 500 μL of the OPA solution was added, mixed, and allowed to react for 5 min at room temperature before the full volume of the sample was injected.

Resolution of derivatized products was performed using a Beckman HPLC equipped with either a 5 μ 4.6 ×150 mM Beckman or 5 μ 4.6×250 mM Microsorb C_{18} Reverse-Phase Column. Methanol and a solution of 80 mM sodium citrate, 20 mM sodium phosphate, with 0.02% sodium azide at pH 6.8 were employed as cosolvent. Gradients were optimized for each reaction condition tested. The fluorescent signals were detected on a Waters 470 scanning fluorescence detector with an excitation wavelength of 344 nM and an emission wavelength of 443 nM.

Quantities of the amino acid enantiomers produced were calculated by integration of peaks from calibration curves of D,L amino acid standards generated over a range of concentrations. For peptide BP1, the initial reaction rate was determined by fitting the total amino acid product for each time-point to a pseudo first order exponential build-up of product. From this fit, the initial rate was extracted. For peptides CBP01-CBP18, the extent of reaction was calculated from the total quantity of amino acid produced. The enantiomeric excess was calculated from the difference in quantity of amino acid enantiomers produced relative to the total quantity of amino acid produced. All samples were performed in duplicate.

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