Coenzyme−Amino Acid Chimeras: New Residues for the Assembly of Functional Proteins

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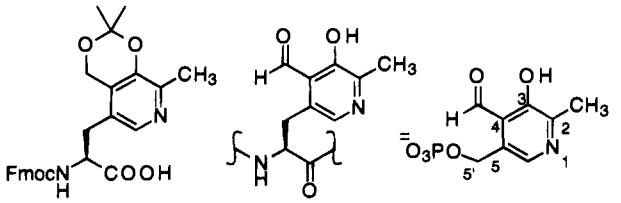
Coenzymes play a unique role in enzyme catalysis by addressing functions not accessible to the coded amino acids.[1] Although this potential could be exploited in the design of new functional proteins, the use of coenzymes is limited by the need for explicit binding sites or amenability to selective chemical modification.[2−4] A unique solution to this problem is found in the pyruvoyl-containing[5] and topaquinone-containing[6] enzymes, wherein post-translational modification transforms residues into covalently bound coenzyme moieties. Acting on this clue from nature, we have formulated a program that aims to develop coenzyme−amino acid chimeras as key components for the next generation of *de novo* designed functional proteins. Herein, we introduce the α-amino acid (*S*)-2-(N^{α}-(9-fluorenylmethyloxycarbonyl)amino)-3-(α^4,3-*O*-isopropylidenepyridox-5-yl)propanoic acid[7] (Fmoc-L-Pol(Iso)-OH, **1**), which has been incorporated into polypeptides *via* solid phase peptide synthesis and converted to the reactive pyridoxal analog (Pal, **2**). A demonstration of enhanced transaminase activity within a semisynthetic protein highlights the utility of this approach in the *de novo* design of functional biomolecules.



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Pyridoxal phosphate (PLP, **3**) catalyzes a variety of reactions involving α-amino acids.[8] Processes that have been studied in *non-peptidyl* systems include transamination,[9,10] racemization,[11] and decarboxylation.[12] Unlike the PLP-dependent enzymes,[1] many models employ polyvalent metal ions to orient and activate the Schiff base intermediate.[13] The Pal residue may, however, facilitate the development of a *peptidyl* system with an intrinsic capacity to activate the reactive intermediate. Strategically, Pol may be introduced into polypeptides by total chemical synthesis or semisynthesis.[14] In the present study, we have chosen a semisynthetic approach wherein the Pal residue is positioned at a judiciously selected site within a ribonuclease S (RNase-S) complex. Herein we demonstrate that the Pal chimera effects the transamination of a substrate amino acid under single turnover conditions, without assistance from metal.

RNase-S complexes are semisynthetic constructs comprising residues 21−124 of native ribonuclease A (S-protein) and an oligopeptide based on residues 1−20 (S-peptide).[15] Synthetic S-peptide derivatives are useful vehicles for the introduction of unnatural residues into the complex.[16,17] Analysis of the crystal structure of RNase-S[18] revealed that a $Phe_8 \rightarrow$ Pol substitution in S-peptide would position the residue in proximity to the general acid−base pair (His_{12}, His_{119})[19] utilized by the native enzyme. The Lys residues in native S-peptide$_{1-14}$ were replaced with either norleucine (Nle)[17] or Gly, to prevent intramolecular Schiff base formation with the coenzyme chimera (Pal_8). Met_{13} was substituted with Nle to ensure compatibility with the requisite oxidation step that follows peptide synthesis without compromising RNase complex formation. Thus, on the basis of these design considerations, S-peptide$_{1-14}$ derivatives C1 and C2 were synthesized by standard Fmoc solid phase synthesis.[20] A generalized ribbon diagram[21] of the proposed RNase-S complex is shown in Figure 1. Selective oxidation of the Pol_8 residue to Pal_8 was accomplished using γ-MnO_2[22] in dimethyl sulfoxide. The Pal-peptides were quantified by the unique UV absorption of the pyridoxal moiety in 0.1 N NaOH,[23] and the mixtures of reduced and oxidized peptides (10−29% Pal-peptide) were assayed directly for transamination.[24]

Residue:	1	5	10	14
SP_{1-14}:	Lys E T A A A	Lys Phe	E R Q H	Met D NH_2
C1:	Nle - - - - - - - -	Gly Pol	- - - - - - -	Nle - - NH_2
C2:	Nle - - - - - - - -	Nle Pol	- - - - - - -	Nle - - NH_2

For each derivative, the dissociation constant (K_D) of the complex formed between S-protein (SP) and the Pol-peptide was measured[25] and used as a reasonable estimate for that of the corresponding Pal system. Pol-peptides C1 and C2 bound S-protein with affinities comparable to that of native S-peptide$_{1-14}$ ($K_{D(SP_{1-14})}$ = 13.3 μM; $K_{D(C1)}$ = 16.0 μM; $K_{D(C2)}$ = 32 μM). The corresponding Pal-peptides were assayed spectroscopically[26] (Figure 2) for their ability to effect the transamination of

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(15) Bovine ribonuclease A is selectively cleaved by subtilisin into S-peptide and S-protein. Noncovalent association of these fragments yields a fully active enzyme complex (RNase-S). S-peptide$_{1-14}$ is also sufficient for complex formation. See: Hofmann, K.; Frances, F. M.; Limetti, M.; Montibeller, J.; Zanetti, G. *J. Am. Chem. Soc.* **1966**, *88*, 3633. Richards, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1958**, *44*, 162.
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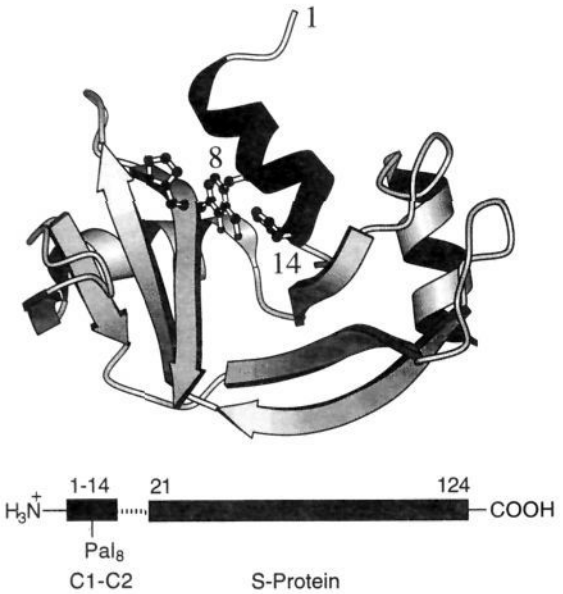

Figure 1. Proposed model for the semisynthetic RNase-S complex incorporating the Pal residue. The S-peptide fragment is highlighted, illustrating the location of the coenzyme chimera (Pal_8) and the general acid−base pair. A schematic of the complex is presented for comparison.

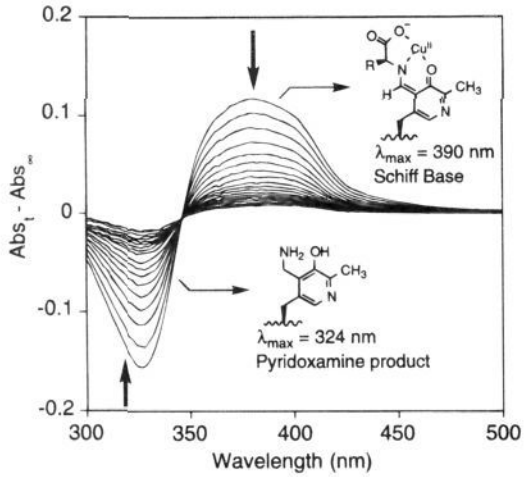

Figure 2. UV−visible difference spectra acquired at 80-min intervals for the Cu^{II}-catalyzed transamination of L-Ala by complex C2−SP at pH 4.0, 25 °C.

L-alanine to pyruvic acid, under single turnover conditions, in the presence and absence of Cu^{II}.[27] The assays were repeated with sufficient S-protein to ensure 90% complex formation with the peptides. The transamination rates of 5′-deoxypyridoxal (DPal) were also measured to evaluate the intrinsic reactivity of the coenzyme moiety.

(24) The unoxidized Pol-peptides are catalytically inactive and do not interfere in the kinetic analysis. The pyridoxamine-peptide product of transamination was dansylated and characterized by mass spectroscopy (e.g., Dns-Pmn-C1, calcd for MH^+ 1849, obsd 1848.6). Higher oxidation yields (30−50%) have been obtained with model hexapeptides, indicating that steric hindrance/aggregation effects might affect the oxidation step. The process is selective in the presence of most residues including serine, threonine, and histidine. Current efforts are focused on the introduction of the coenzyme chimera into polypeptides in the pyridoxamine oxidation state, which will avoid post-synthesis modification.

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Table 1. Kinetic Data for the Transamination of L-Ala by the Various Pal Systems

Pal system	$k_{obsd} \times 10^5$ (min^{-1})		$t_{1/2}$ (h)
	+Cu^{II}	−Cu^{II}	−Cu^{II}
C1	282	71	16.3
C1−SP	510	1288	0.9
C2	289	95	12.1
C2−SP	198	107	10.8

The kinetic data for Pal-peptides C1 and C2 are summarized in Table 1. The activities of both peptides were influenced by S-protein, thus implicating the RNase complex as the prime reactive species under the reaction conditions. The best transamination rates were observed with the C1−SP complex and probably resulted from a favorable orientation of the Schiff base intermediate within the protein. Considerably slower rates were observed for the *uncomplexed* Pal-peptides. The diminished reactivity of the C1−SP complex in the presence of Cu^{II} reflects the lower activating capacity of the metal ion. In addition, copper may inhibit the transamination process by chelating the substrate and decreasing the effective concentration of the reactive species (free amine). This effect was less pronounced in the uncomplexed Pal-peptides, wherein chelation of the aldimine was mandatory for activation.

Replacement of a single residue ($Gly_7 \rightarrow Nle_7$) in peptide C2 further highlighted the influence of peptide architecture on reactivity. Complex formation in peptide C2 was accompanied by a 12-fold *drop* in transaminase activity relative to the C1−SP complex. Computer modeling suggests that this diminished reactivity might be due to occlusion of the coenzyme moiety (Pal_8) in the C2−SP complex by Nle_7. Unlike the Pal-peptides, DPal consistently exhibited incomplete transamination (~50%) and low reactivities, which were not significantly influenced by metal ions or S-protein ($k_{obs} < 91 \times 10^{-5}$ min^{-1} for all cases).

The semisynthetic proteins described in this communication demonstrate the viability of the coenzyme−amino acid chimeras in the creation of new functional biomolecules. The utilization of these amino acids at variable positions within the polypeptide sequence allows future design efforts to focus on harnessing auxiliary residues within the protein scaffold for selectivity and catalytic turnover. The availability of these chimeras therefore opens new avenues in *de novo* protein design.

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Supplementary Material Available: The synthetic scheme for Fmoc-L-Pol(Iso)-OH and peptides C1 and C2, details of the binding experiments, and transamination assays (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(27) Assays were performed at 25 °C in 0.1−1.0 M acetate buffer, pH 4.0, $\mu = 0.29$ (KCl). Initial concentration of reactants: Pal-peptide, 1.0×10^{-4} M; $CuCl_2$, 1.0×10^{-4} M; L-Ala, 1.0 M. Metal-free runs were carried out in the presence of 1 mM EDTA (instead of $CuCl_2$) to mask the effect of traces of contaminating metal ions. See: Kondo, H.; Kikuchi, J.; Sunamoto, J. *Tetrahedron Lett.* **1983**, *24*, 2403. Spectra were acquired every 10 or 20 min, for 6−60 h. The conversion of Schiff base to pyridoxamine-peptide followed first-order kinetics.