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SITE-SPECIFIC INCORPORATION OF PHOTOISOMERIZABLE AZOBENZENE GROUPS INTO RIBONUCLEASE S

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Abstract: Syntheses of S-peptide analogues bearing phenylazophenylalanine (Pap) residues at positions 4, 8, and 11 are described. Noncovalent reassociation of the Pap-4 and Pap-11 peptides with S-protein reconstitutes ribonuclease activity. Photoisomerization of the Pap-4 peptide is found to modulate the enzyme activity.

Introduction:

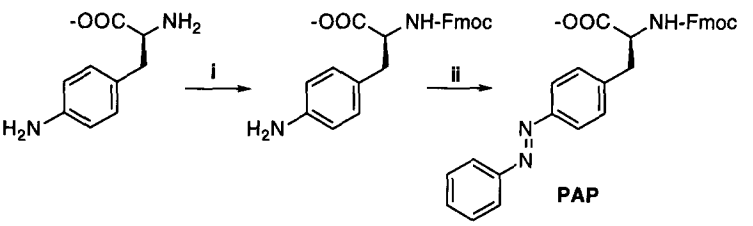
Photoregulation of biologically active compounds can provide important means for the study of complex living systems in situ.[1] Reversible photoregulation, in particular, could enable detailed studies of timing and rhythms in biological systems.[1-3]

Several approaches to the reversible photoregulation of enzymes have been employed, including the design of photoisomerizable inhibitors, casting the enzyme in a photoisomerizable matrix, and chemical modification of the enzyme itself.[4-7] Most studies involving chemical modification of the enzyme have employed nonspecific reagents that couple many photochromic units to undefined sites on the protein. Rational design of photoswitching would be facilitated if photochromic groups could be incorporated site-specifically at key sites in the enzyme structure. Recently, Ueda et al.[8] have reported the site-specific inclusion of phenylazophenylalanine (Pap) at position 3 of amidinated phospholipase A2. This was accomplished by Edman degradation of the full length protein followed by chemical coupling of a synthetic tripeptide containing Pap. Coupling required that all lysine residues on the protein be blocked.

We wish to report here the site-specific incorporation of Pap at three sites within the sequence of ribonuclease A. We take advantage of a useful property of the enzyme (ribonuclease can be cleaved specifically into two parts with subtilisin—an N-terminal peptide termed the S-peptide and the remainder called the S-protein). Separately, these two parts are inactive since each part contributes residues to the enzyme active site. Noncovalent reassociation of the parts, however, can completely restore enzyme activity.[9] Chemical synthesis of S-peptide analogues provides a convenient means for introducing nonnatural amino acids.[9,10] In addition, the well-defined structure of ribonuclease should facilitate attempts to optimize photoswitching of enzyme activity.[11,12]

Synthesis:

The photochromic amino acid Pap was prepared via a modification of the method reported by Goodman and Kossoy.[13] First *p*-amino-phenylalanine was protected at the α amino group using 9-fluorenylmethylsuccinimidyl carbonate (Fmoc-OSu).[14] The protected compound was then treated with 1.5 equiv of nitrosobenzene in 5% acetic acid in methanol and stirred for 30 h at 70 °C.



i: Fmoc-OSu, H_2O/DMF/Na_2CO_3 ii: nitrosobenzene, glacial HAc/MeOH

The reaction mixture was added to water, the precipitate collected, and then purified by silica column chromatography eluting with a solution of ethyl acetate:petroleum ether:methanol (3:2:2.8). The orange coloured product (185 mg, yield 60.6%) was collected. High-resolution FABMS: MH^+ $C_{30}H_{26}N_3O_4$, Calcd: 492.1923, Found: 492.1928.

The following peptides, corresponding to ribonuclease A residues 4–15, were then synthesized:

RNAse Seq#	4 5 6 7 8 9 10 11 12 13 14 15
native	Ac-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-NH_2
pap-4	Ac-Pap-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-NH_2
pap-8	Ac-Ala-Ala-Ala-Lys-Pap-Glu-Arg-Gln-His-Met-Asp-Ser-NH_2
pap-11	Ac-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Pap-His-Met-Asp-Ser-NH_2

Standard solid-phase Fmoc-based peptide synthesis methods were employed.[15] Peptides were constructed on a Pal-resin: (capacity 0.55 mmol/g) (Advanced ChemTech, Louisville, KY). Coupling used 3 equiv HATU [O-(7-Azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; 6 equiv DIPEA (N,N-Diisopropylethylamine), 3 equiv amino acid. Peptides were purified by HPLC (Zorbax SB-C18 column 9.4 × 250 mm, A: 0.1% TFA in acetonitrile, B: 0.1% TFA in water, gradient of 5–95% A in 90 min) and analyzed by HPLC and ESI-MS. The purity by HPLC >95%. All peptides had the expected mass.

Results and Discussion:

Peptides were added to purified S-protein (Sigma) and the rate of RNA hydrolysis was assayed by the Kunitz method (Fig. 1).[16] Enzyme activity increased as peptide was added until a maximum activity was reached. This occurs when all the S-protein has bound peptide. *Trans*-to-*cis* photoisomerization of Pap peptides was accomplished using a nitrogen laser; reversion to the *trans* form was effected by exposing the peptide solution to diffuse sunlight for 1–2 minutes. All peptides showed reversible photochromic behaviour (Fig. 2).[17]

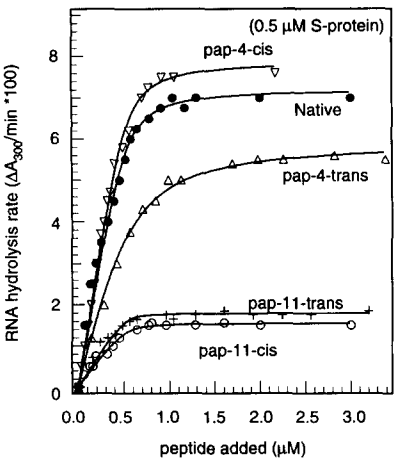


Figure 1. Hydrolysis rate vs. concentration of peptide added. (0.1 M Na acetate buffer, pH 5.0, 50 μg/mL total yeast RNA).

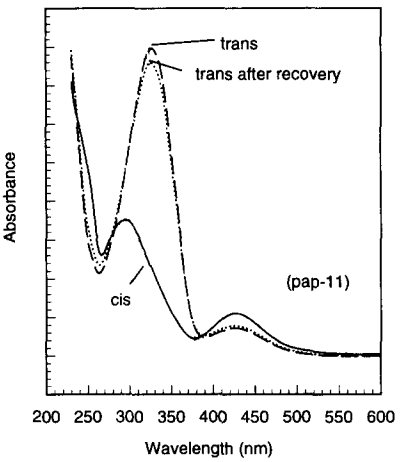


Figure 2. Reversible photochromic behaviour of the Pap-11 peptide in aqueous solution.

No ribonuclease activity was seen when excess Pap-8 peptide was added to S-protein suggesting that either a protein-peptide complex did not form or was inactive. The Pap-11 peptide was able to restore activity to approximately the same extent in either *cis* or *trans* states. The concentration of Pap-11 peptide required for maximum activity was similar to the native case indicating that the Gln-11 to Pap-11 mutation has little effect on peptide binding to S-protein in either *cis* or *trans* forms. Both forms of the Pap-11 peptide exhibit a maximum activity about fourfold less than that seen with the native peptide.

The Pap-4 *cis* peptide restored activity to a maximum level somewhat higher than that of the native peptide at the same concentration. The Pap-4 *trans* peptide, on the other hand, showed a maximum activity about 25% less than the Pap-4 *cis* peptide and a slightly lower affinity for the S-protein.

The low sensitivity of the Kunitz assay makes accurate determination of V_{max} and Km values of the mutant enzymes difficult; we are currently preparing fluorogenic substrates that should make a full kinetic analysis possible. Mono- and dinucleotide substrates now employed as substrates[9,18] are not expected to be affected by Pap photoisomerization in the same way as RNA polymers since the conformational change (at least in the Pap-4 case) is occurring at a site remote from the scissile bond.

To maintain reversibility of photoswitching, trapping of one or other photoisomeric state, through a strong binding interaction for instance, should be avoided. Photoregulation of substrate access to the active site of an enzyme could, in principle, achieve this objective. The availability of high-resolution crystal structures of free and substrate-bound ribonuclease[19] should facilitate the design process as well as the interpretation of changes in activity upon photoisomerization (or the lack of a change as with Pap-11) in terms of the structure and dynamics of the modified protein.

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