The Mechanism of Self-Catalyzed Site-Selective Functionalization of a Designed Helix−Loop−Helix Motif

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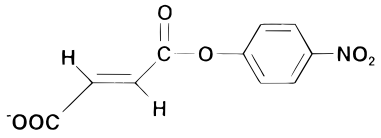
Designed polypeptides[1,2] with supersecondary structure have great potential in the engineering of tailor-made catalysts. Several examples of catalytically active polypeptides have been reported, but only in a few cases have studies of structure and reactivity been possible.[3,4] The function of these polypeptides is, however, still only partly understood. In this communication we wish to report on the catalytic mechanism of the reaction between RA-42, a designed polypeptide with 42 amino acids, Figure 1, and *p*-nitrophenyl fumarate (I) to form an amide at the side chain of ornithine-15 of RA-42.

The design of RA-42 was based on the solution structure of a template polypeptide SA-42, a helix-loop-helix dimer.[5] RA-42, too, folds in solution into a hairpin helix−loop−helix motif, and the solution structure has been determined by NMR and CD spectroscopy.[3] In short, the CD spectrum showed the features that are typical of helical peptides and the measured mean residue ellipticity, −18 800 deg cm^2 dmol^{-1} in aqueous solution and −22 900 deg cm^2 dmol^{-1} in 10 vol % TFE, showed a large helical content. The mean residue ellipticity was independent of concentration in the range used for reaction mechanistic studies. The ^{1}H NMR NOESY spectrum showed through-space connectivity between the methyl groups of the hydrophobic residues in helix I and the aromatic protons of phenylalanines in helix II, showing the formation of the hairpin motif.



I

The reaction center of RA-42 includes His-11, Orn-15, and Orn-34. His-11 catalyzes the acylation of the side chain of ornithine-15 in a self-functionalization reaction[3] leaving other amino groups unfunctionalized. The reaction has been studied at 0.5−1 mM concentration of peptide. The second-order rate constant for the reaction of RA-42 is 2.8 × 10^{-2} dm^3 mol^{-1} s^{-1} in 10 vol % trifluoroethanol (TFE) at pH 5.9[3] which is more than 10^3 times larger than that of the comparable reaction between ethylamine and *p*-nitrophenyl acetate.[6] The His-11 catalyzed reaction is 8.3 times faster than that catalyzed by 4-methylimidazole at pH 5.9 in 10 vol % TFE. In aqueous solution the second-order rate constants are 5.1 ± 0.1 × 10^{-2} M^{-1} s^{-1} at pH 5.9 for RA-42 and 1.05 ± 0.02 × 10^{-2} M^{-1} s^{-1} for 4-methylimidazole. The observed rate enhancement is therefore almost the same as in 10 vol % TFE.

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N-Aib-A-D-Nle-F-A-A-I-K-**H**-L-A-E-**Orn**-Nle-Aib-A-K
1 19
G-P-V-D
20 23
G-Aib-R-A-F-A-E-F-**Orn**-K-A-L-Q-E-A-Nle-Q-A-Aib
42 24

RA-42

Figure 1. Amino acid sequence of RA-42. The residues presented underlined in bold are the ones designed to constitute the binding site. The one letter code for amino acids is used where A is Ala, D is Asp, E is Glu, F is Phe, G is Gly, H is His, I is Ile, K is Lys, L is Leu, N is Asn, P is Pro, Q is Gln, R is Arg, V is Val. Aib is α-aminoisobutyric acid and Nle is norleucine.

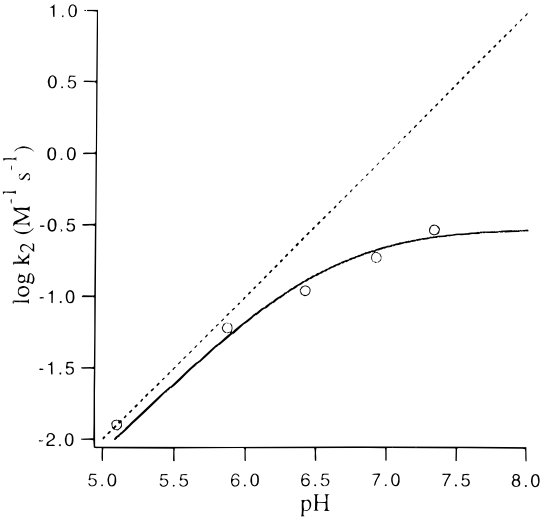

Figure 2. The logarithm of the second-order rate constants for the release of *p*-nitrophenol vs pH. The second-order rate constants have been calculated from the observed pseudo-first-order rate constants by division with the total concentration of peptide. Each kinetic point is the average of 2 runs, and the full line shows the expected dependence on pH of a reaction that is catalyzed by the unprotonated form of an acid with a pK_a of 6.4. The experimental pH interval from 5 to 7.5 was chosen due to experimental considerations. At pH 5 the reaction is very slow, at high pH the background reaction becomes very fast since the logarithm of its reaction rate constant is proportional to pH, whereas the RA-42 catalyzed reaction has a limiting value. The dotted line shows the expected dependence on pH of the rate constant of a reaction catalyzed by the deprotonated form of ornithine with a pK_a of 10.4.

For the reaction between RA-42 and mono-*p*-nitrophenyl fumarate (I) the electrospray mass spectrum and the NMR spectrum of the reaction products show that an amide is formed at the side chain of Orn-15.[3]

The pH dependence of the second-order rate constants for the acyl-transfer reaction between mono-*p*-nitrophenyl fumarate (I) and RA-42 was measured in 50 mM Bis-Tris buffer at 290.2 K, Figure 2. The reaction is first-order with respect to the concentration of peptide. The second-order rate constants increase with pH which shows that the reaction depends on an amino acid residue in its deprotonated form. If the products were formed in a direct reaction between Orn-15 and I, the logarithm of the second-order rate constants would show a linear dependence on pH in the range from 5 to 8, as the pK_a of the side chain of the ornithine residue is probably between 10 and 11.[7] The observed pH profile rules out this mechanism. The plot of the logarithm of the second-order rate constants versus pH in the interval from 5.9 to 7.3 shows that the reaction depends on an amino acid residue with a pK_a close to 6.5.

The amino acid sequence of RA-42 contains the ionizable residues Asp, Glu, Arg, Orn, Lys, and His as well as the C-terminal carboxylic acid and the N-terminal amino group of

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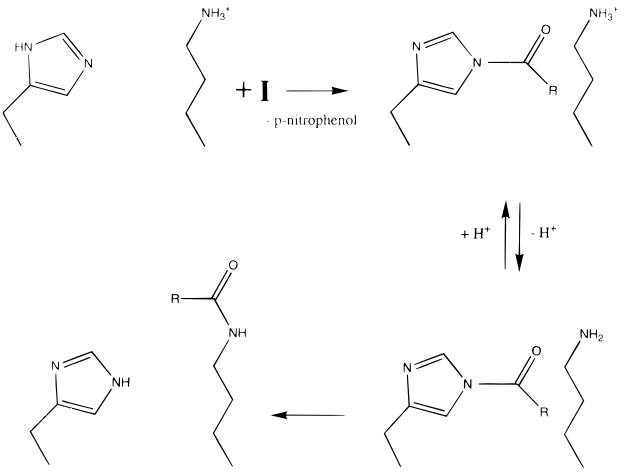

Figure 3. The reaction mechanism of RA-42 self-catalyzed site selective functionalization. An initial attack by the side chain of His-11 on the carbonyl carbon of I leads to the formation of an acyl intermediate with the liberation of *p*-nitrophenol. The acyl intermediate is a reactive acylimidazolide. In the second step the acyl group is transferred to the deprotonated form of the side chain of Orn-15.

the peptide backbone. Typical pK_a's of ionizable residues in small peptides[7] have been determined and are 4.0 for Asp, 4.5 for Glu, 6.4 for His, 10.4 for Lys (and probably for Orn), 12.4 for Arg, 3.6 for the C-terminal carboxylic acid, and 7.8 for the N-terminal amino group. The pK_a of the corresponding acid of the side chain of His-11 was determined by ^{1}H NMR spectroscopy, and it was found to be 6.5 in aqueous solution. The only amino acid residue in RA-42 with a pK_a value close to 6.5 is His-11. Therefore an initial acyl-transfer reaction of I by initial attack of His-11 gives rise to an acyl intermediate, and a reactive imidazolide is formed. The observed pH dependence also shows that the first step is rate limiting.

The pK_a of the 4-methylimidazolium ion has been determined, and it is 7.9 in aqueous solution at 303 K. The second-order rate constant for the 4-methylimidazole catalyzed reaction in the pH independent region can then be calculated from that measured at pH 5.9, and it is 1.33 M^{-1} s^{-1}. The second-order rate constant for the reaction of RA-42 in the pH independent region that was calculated from the measured pK_a of 6.5, and the second-order rate constant at pH 5.9 is 0.305 M^{-1} s^{-1}, Figures 2.

The second-order rate constant of 4-methylimidazole is, based on the Bronstedt relation 1, expected to be 13.2 times larger than that of RA-42 since β is

$$\log k_2 = \beta \times \mathrm{p}K_a + A \qquad (1)$$

0.8 for imidazole catalyzed hydrolysis of *p*-nitrophenyl acetate.[8] It is found to be only 4.4 times larger, and RA-42 therefore catalyzes the acylation of His-11 by a factor of 3 most likely by stabilization of the developing oxyanion in the transition state by the side chain of Orn-15. The observed rate enhancement of RA-42 over 4-methylimidazole at pH 5.9 is close to a factor of 5, and the rate enhancement beyond the factor of 3 mentioned above is due to the fact that the stronger nucleophile 4-methylimidazole is protonated to a larger extent than the side chain of His-11. At pH 5.9 the concentration of unprotonated His-11 is 21 times larger than that of unprotonated 4-methylimidazole if the total concentration of peptide is the same as the total concentration of 4-methylimidazole. If the rate enhancement were due to the concentration effect alone RA-42 would be a more efficient catalyst by a factor of 1.6 (21/13.2) at pH 5.85. The observed rate enhancement by RA-42 is therefore due to transition-state stabilization and pK_a depression, two factors commonly encountered in naturally occurring catalysts.

The suggested role of the flanking ornithines is supported by the observation that the second-order rate constant for the reaction of SA-42, that has no flanking ornithines, is very close to that of the 4-methylimidazole catalyzed reaction.[3] Since no evidence for saturation kinetics has been observed, the binding interaction with the ornithines must take place in the transition state.

The final reaction product is an amide formed at the side chain of Orn-15, and the second step of the reaction is therefore an acyl group transfer in a fast intramolecular reaction from His-11 to the deprotonated form of Orn-15. The rate enhancement of the intramolecular reaction cannot be measured since the acylation of His-11 is the rate limiting step. It is, however, high because no trace of the acyl intermediate could be detected in the ^{1}H NMR spectrum recorded under reaction conditions (10 vol % TFE 90% aqueous Bis-Tris buffer in H_2O/D_2O 90/10, pH 5, 293 K). A fast intramolecular reaction without accumulation of an intermediate is also consistent with the observed first-order kinetics of RA-42.

The histidine residue introduces a functional group at the side chain of Orn-15 in a highly selective reaction after which free histidine is regenerated. The other ornithine in RA-42, Orn-34, does not form an amide, and it has no neighboring histidine residue. Lys-10 which is next to His-11 in the sequence and therefore close in space also does not form an amide under the reaction conditions. The observed selectivity of the reaction center implies that not only can histidine be used to introduce new functionality in folded polypeptides, but it can also be used for the stepwise introduction of diverse functionality, provided that differential reactivity in the side chains can be engineered. Such reactions have great potential in site-selective functionalizations and in the construction of functionalized polypeptides, e.g., novel catalysts. The reaction may be used to introduce residues that will not survive under the reaction conditions of peptide synthesis or that will not be reactive enough due to steric hindrance. Novel branched polypeptide structures are also possible if amino acid residues or peptides can be introduced. Since the histidine is regenerated it can also be designed to participate in the active site of an engineered catalyst.

The detailed understanding of the reaction mechanism and its structural basis that is presented here is unique for designed polypeptides with supersecondary structure, and it is of general interest in the design of catalytically active polypeptides. It provides the necessary platform for further development of RA-42 and other designed polypeptides into more efficient catalysts, particularly catalysts capable of catalyzing stereoselective reactions.

The design and synthesis of RA-42 have been described in detail previously.[3,5] The kinetic measurements were carried out using a Cary 4 UV−vis spectrometer equipped with a Cary temperature controller. The pK_a determination was carried out on a Varian Unity 500 NMR spectrometer operating at 290 K by measuring the chemical shifts of the histidine aromatic protons as a function of pH.

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