Enzyme Mimics

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Downsizing of Enzymes by Chemical Methods: Arginine Mimics with Low pK_a Values Increase the Rates of Hydrolysis of RNA Model Compounds

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The downsizing of enzymes is an attractive goal.[1] It increases our understanding of enzyme function and enables many applications in biotechnology. The fine-tuning of amino acid pK_a values to match reaction mechanisms, which are controlled in proteins by the active-site molecular environment, is a critically important aspect of enzyme catalysis that is difficult to mimic in model peptides. The catalytic residues are exposed to solvent, and effects on pK_a values by neighboring groups are limited to an increase or decrease of approximately one unit.[2] In contrast, the chemical synthesis of amino acids with non-natural side chains can be useful for the control of dissociation constants with high accuracy and offers a fast and robust route to improved catalysts. Herein we report on the use of a synthetic arginine mimic, a guanidiniocarbonyl pyrrole derivative named Gcp, with a pK_a value of 6–7 in aqueous solution. This value is several units lower than that of Arg.[3] Gcp was introduced in a polypeptide scaffold to provide transition-state stabilization and general-acid/general-base catalysis in reactions that mimic RNA hydrolysis.

Phosphodiesters are extremely stable towards hydrolysis, and the mechanism for the catalysis of RNA hydrolysis by enzymes and ribozymes has been studied thoroughly.[4] Enzymes that have evolved to catalyze phosphodiester hydrolysis are among the most efficient known; they show rate enhancements of eighteen orders of magnitude or more.[5] Ribonucleases provide general-acid and general-base catalysis as well as substrate binding and transition-state stabilization. Numerous model systems ranging from metalloenzyme mimics[6] and metallocomplexes[7] to polyamines[8] have been designed to mimic all or part of the catalytic machinery, but full ribonuclease activity remains an elusive goal, most likely because of the difficulties encountered in combining all of the necessary catalytic components in the correct orientation in a small catalyst.

We previously reported a helix–loop–helix motif (HNI, Figure 1) based on commonly occurring amino acids and capable of catalyzing the hydrolysis of RNA models with rate enhancements of two orders of magnitude or more.[9] HNI forms a helix–loop–helix motif with two His residues in opposite corners of an approximately square-shaped active site and two Arg residues in the remaining two corners. The imidazole side chains most likely act as general-acid and/or general-base catalysts. The flanking arginine residues were introduced to bind the negatively charged phosphate ester and the even more negatively charged transition state by electrostatic interactions as well as by hydrogen bonding. Since the transition state is more negatively charged than the substrate, we expect it to be bound more strongly than the substrate and expect transition-state binding to contribute significantly to catalysis.

Although arginine residues are considered to be good binding groups for phosphates, the pK_a values of the guanidino group and phosphate groups are not well-matched. The arginine residue has a pK_a value of more than 12 in short peptides,[10] whereas the pK_a value of the phosphate group in a phosphodiester is approximately 1.3.[11] It seemed very likely that an arginine mimic with a pK_a value as low as the pH value of the solvent for the reaction would offer considerable improvements in catalytic efficiency in phosphoryl-transfer reactions through improved hydrogen bonding. The concept has previously been demonstrated elegantly in nonpeptidic catalysts.[12] Furthermore, general-acid and general-base catalysis by His residues is hampered by the short side chain and short reach relative to the distances between residues on the surface of a helix–loop–helix motif. We introduced the Gcp residue in HNI to replace both Arg residues (JL1), both His residues (JL2), and both Arg as well as both His residues (JL3; Figure 1). The pK_a value of the guanidinium residue of this non-natural amino acid in aqueous solvents is approximately 6–7, close to that of His.[13] The guanidiniocarbonyl pyrrole cation has been shown to be an excellent receptor for carboxylates with dissociation constants in the millimolar range, even in aqueous solvents.[14]

The peptides were synthesized by solid-phase peptide synthesis as reported previously for HNI, but by attaching the guanidiniocarbonyl pyrrole group to selectively deprotected ornithine residues by using amide-coupling chemistry.[15,16] After deprotection and cleavage from the solid support, the

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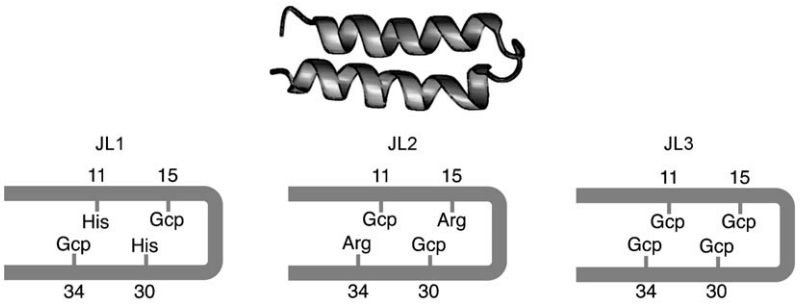

JL1 N-A-A-D-Nle-E-A-A-I-K-H-L-A-R-Gcp-Nle-A-A-K-G-P-V-D-A-A-Q-Nle-A-E-H-L-A-R-Gcp-F-E-A-F-A-R-A-G

JL2 N-A-A-D-Nle-E-A-A-I-K-Gcp-L-A-R-R-Nle-A-A-K-G-P-V-D-A-A-Q-Nle-A-E-Gcp-L-A-R-R-F-E-A-F-A-R-A-G

JL3 N-A-A-D-Nle-R-A-A-I-K-Gcp-L-A-R-Gcp-Nle-A-A-K-G-P-V-K-A-A-Q-Nle-A-A-Gcp-L-A-R-Gcp-F-A-A-F-A-R-A-G

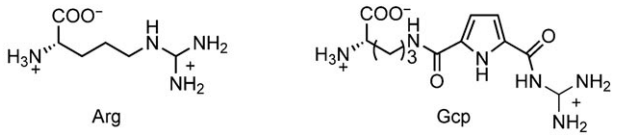

Figure 1. Illustration of the active sites of JL1, JL2, and JL3, the polypeptide sequences in the one-letter code (Nle is norleucine and Gcp is the guanidiniocarbonyl pyrrole amino acid), and the structures of the Arg and Gcp residues. The peptides form helix–loop–helix structures which dimerize in solution.

peptides were obtained in 10–20% yield after reversed-phase HPLC purification and identified by MALDI-TOF MS.

The mean residue ellipticities at 222 nm, Θ_{222}, recorded at a concentration of 50–60 μM, were −16800, −18400, −22500, and −24300 deg cm^2 dmol^{-1} for HNI, JL1, JL2, and JL3, respectively (see Figure S6 in the Supporting Information). The measured mean residue ellipticities suggested that the peptides formed helix–loop–helix dimers at these concentrations, as the helicity of monomers is known to be very low for similar sequences.

We studied the phosphoryl-transfer reaction of 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP, **1**), an activated substrate well-accepted to undergo cleavage by general-base catalysis, as this reaction mimics the first step of RNA hydrolysis. HPNP undergoes transphosphorylation in an intramolecular reaction to form a cyclic phosphorane with the release of the *p*-nitrophenolate anion. The release of this anion can be monitored conveniently by UV spectroscopy for the determination of rate constants.

The hydrolysis reaction in the presence of each peptide was shown to be first-order in the substrate (see Figure S4 in the Supporting Information), and the reaction is first-order in the peptide, since plots of pseudo-first-order rate constants versus peptide concentration were linear.

In JL1, both Arg residues of HNI were replaced with Gcp. JL1 catalyzed the transesterification reaction of **1** with a second-order rate constant of 2.4×10^{-4} M^{-1} s^{-1}, which is one order of magnitude larger than the second-order rate constant of the HNI-catalyzed reaction (2.2×10^{-5} M^{-1} s^{-1}) and two orders of magnitude larger than that of the imidazole-catalyzed reaction at pH 7.0 (Table 1). In JL2, both His residues of HNI were replaced with Gcp, and both Arg residues remained. The rate enhancement in comparison to catalysis by HNI was larger than that observed with JL1, but only by a factor of 1.5. Gcp was thus shown to be able to replace both the Arg and the His residues of HNI, and to provide a rate enhancement of an order of magnitude in each case. In JL3, both the Arg and the His residues of the catalytic site of HNI were replaced with Gcp. JL3 catalyzed the reaction with rate enhancements of a factor of 150 with respect to the HNI-catalyzed reaction and a factor of 1500, or more than three orders of magnitude, with respect to the reaction catalyzed by imidazole: a dramatic improvement for such a small change in the polypeptide sequence.

The only differences between the sequence of HNI and those of JL1, JL2, and JL3 were that two Arg residues and two His residues in the putative reactive site of HNI were replaced by Gcp residues; the observed rate enhancements were thus due exclusively to the activity of those amino acids. Since the Arg residues in HNI are expected to provide transition-state stabilization by binding to the phosphate group, the Gcp groups most likely have the same function in JL1. One can assume that the better matching of pK_a values with the guanidiniocarbonyl pyrrole cation and the phosphate group enables an improvement in catalysis by an order of magnitude.[17] Furthermore, the replacement of His residues with Gcp to form JL2 gave rise to an even larger rate enhancement. This result suggests that Gcp is also capable of general-base and/or general-acid catalysis. The introduction of four Gcp residues to form JL3, and thus the replacement of the entire active site of HNI, provided strong evidence that Gcp is able to fulfill the functions of both Arg and His, and indeed capable of stronger transition-state binding than that of Arg and more efficient general-acid and/or general-base catalysis than that of His.

Table 1: Second-order rate constants, k_2 [10^{-4} M^{-1} s^{-1}], for the phosphoryl transfer of HPNP (**1**) and uridine-3′-2,2,2,-trichloroethyl phosphate (**2**).[a]

	Imidazole	HN1	JL1	JL2	JL3
1[b]	0.022	0.22	2.4	3.6	33
2[c]	0.017	0.87	1.4	2.3	11[d]

[a] Reaction conditions: 50 mM Bis-Tris, pH 7.0, 313 K. [b] Substrate concentration: 10 mM. [c] Substrate concentration: 2 mM. [d] k_{cat}/K_M.

Although HPNP serves as an interesting model substrate for RNA hydrolysis, uridine-3′-2,2,2,-trichloroethyl phosphate (**2**) is a more realistic mimic with a leaving-group

pK_a value of 12.5 and a structural element that is a true RNA nucleotide. The pK_a value of the 5′-OH group of ribose is 14.5. We investigated the catalysis of the cyclization of **2**.

The JL3-catalyzed reaction of **2** was found to follow saturation kinetics, the hallmark of enzyme catalysis (Figure 2). The Eadie–Hofstee plot shown in Figure 2 provided a k_{cat}/K_M ratio of $1.1\times10^{-3}\,M^{-1}s^{-1}$, a k_{cat} value of $2.8\times10^{-5}\,s^{-1}$, and a K_M value of 26 mM. Although the second-order rate constant of the HNI-catalyzed reaction and the k_{cat}/K_M ratio for JL3 catalysis are not strictly comparable, k_{cat}/K_M can be considered as an apparent second-order rate constant. A comparison between JL3 and HNI shows that JL3 is a more efficient catalyst by more than an order of magnitude. The observation that JL3 binds the substrate in a productive mode proves that it also binds the transition state. The large rate enhancement suggests that the transition state is bound more strongly than the substrate.

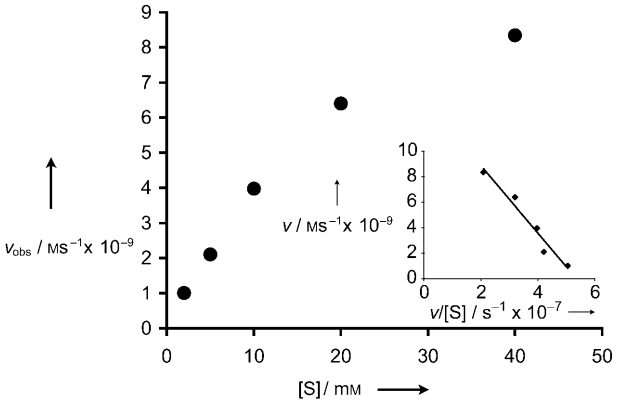

Figure 2. Plot of the observed reaction rate versus the concentration of the substrate for the JL3-catalyzed cyclization of **2** in 50 mM Bis-Tris buffer at pH 7.0 and 313 K: The reaction follows saturation kinetics. The concentration of JL3 was 500 μM. Insert: Eadie–Hofstee plot for the determination of the Michaelis–Menten parameters.

To elucidate the reaction mechanism, we determined the kinetic solvent isotope effect k_{H_2O}/k_{D_2O} at pH 7 and found it to be 1.7, which shows that a proton is in flight in the transition state. We determined the pH dependence in the pH range 4–7.5 (Figure 3) and concluded that catalysis depends on a residue in its unprotonated form with a pK_a value of around 5. Together these observations are compatible with general-base catalysis. A pK_a value of around 5 is not unreasonable for a Gcp residue in the presence of other protonated Gcp groups. In view of the fact that the large rate enhancements are associated with the introduction of Gcp residues, we conclude that a Gcp residue with a depressed pK_a value provides general-base catalysis, whereas flanking Gcp groups are involved in transition-state binding as well as substrate binding.

Non-natural amino acids with tailored properties can thus be used to enhance catalytic activity in enzyme models to enable the downsizing of functional proteins to smaller and less vulnerable structures.

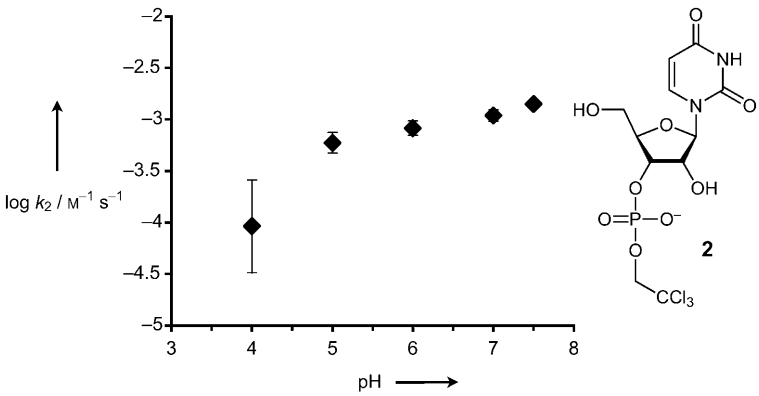

Figure 3. Dependence of the catalysis of the cyclization of **2** by JL3 on the pH value.

Experimental Section

Peptides were prepared according to standard 9-fluorenylmethoxycarbonyl (Fmoc) protocols, with the exception of the Gcp side chains, which were introduced after the selective deprotection of *N*-allyloxycarbonyl (Aloc) ornithine residues in the full-length peptides on the solid phase.[15] Peptide solutions for kinetic measurements were prepared by diluting stock solutions to final concentrations of 2 mM in 50 mM Bis-Tris buffer containing 0.1 % NaN_3 (Bis-Tris = 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol). The pH value was adjusted by the addition of NaOH. Accurate aliquotes from peptide stock solutions were transferred to Eppendorf tubes to give, upon addition of the substrate, final peptide concentrations of 0.4, 0.6, and 0.8 mM in 50 mM Bis-Tris buffer with 0.1 % (w/v) NaN_3. Stock solutions of HPNP (4, 20, and 40 mM in 50 mM Bis-Tris buffer) were freshly prepared before addition to the reaction mixture. The samples were transferred to 0.1 cm cuvettes equipped with teflon stoppers to avoid the evaporation of solvent. Each kinetic experiment was carried out twice. The cuvettes were allowed to reach the temperature of the heating block (313 K) before the measurements were started. The pH value was checked before and after each kinetic measurement and was constant within experimental error. The concentration of the *p*-nitrophenolate ion as a function of time was determined at 405 nm with a Varian Cary 100 Bio spectrometer. The concentration was determined from the absorption by using an extinction coefficient of 18700 $cm^{-1}M^{-1}$ and the degree of ionization at the pH value of the reaction mixture. The value v_{obs} was determined for each peptide concentration, and plots of v_{obs} versus the concentration provided the second-order rate constant, k_2 (see Figure S5 in the Supporting Information). A similar setup was used for experiments with imidazole as the catalyst.

CD spectra were recorded at 298 K with a JASCO J-810 spectropolarimeter.

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[1] A. J. Kirby, *Angew. Chem.* **1994**, *106*, 573–576; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 551–553.

[2] K. S. Broo, L. Brive, R. S. Sott, L. Baltzer, *Folding Des.* **1998**, *3*, 303 – 312.
[3] C. Schmuck, L. Geiger, *Chem. Commun.* **2005**, 772 – 774.
[4] T. Lönnberg, H. Lönnberg, *Curr. Opin. Chem. Biol.* **2005**, *9*, 665 – 673; X. Lopez, D. M. York, A. Dejaegere, M. Karplus, *Int. J. Quantum Chem.* **2002**, *86*, 10 – 26.
[5] A. Radzicka, R. Wolfenden, *Science* **1995**, *267*, 90 – 93.
[6] A. Scarso, U. Scheffer, M. W. Göbel, Q. B. Broxterman, B. Kaptein, F. Formaggio, C. Toniolo, P. Scrimin, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5144 – 5149; P. Rossi, F. Felluga, P. Tecilla, F. Formaggio, M. Crisma, C. Toniolo, P. Scrimin, *J. Am. Chem. Soc.* **1999**, *121*, 6948 – 6949.
[7] F. Manea, F. Houillon, L. Pasquato, P. Scrimin, *Angew. Chem.* **2004**, *116*, 6291 – 6295; *Angew. Chem. Int. Ed.* **2004**, *43*, 6165 – 6169; O. Iranzo, T. Elmer, J. P. Richard, J. R. Morrow, *Inorg. Chem.* **2003**, *42*, 7737 – 7746; G. Feng, D. Natale, R. Prabaharan, J. C. Mareque-Rivas, N. H. Williams, *Angew. Chem.* **2006**, *118*, 7214 – 7217; *Angew. Chem. Int. Ed.* **2006**, *45*, 7056 – 7059; M. Jagoda, S. Warzeska, H. Pritzkow, H. Wadepohl, P. Imhof, J. C. Smith, R. Krämer, *J. Am. Chem. Soc.* **2005**, *127*, 15061 – 15070; T. Gunnlaugsson, R. J. H. Davies, M. Nieuwenhuyzen, C. S. Stevenson, R. Viguier, S. Mulready, *Chem. Commun.* **2002**, 2136 – 2137; M. Kalesse, A. Loos, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2063 – 2068.
[8] A. Clouet, T. Darbre, J.-L. Reymond, *Adv. Synth. Catal.* **2004**, *346*, 1195 – 1204; F. Avenier, J. B. Domingos, L. D. Van Vliet, F. Hollfelder, *J. Am. Chem. Soc.* **2007**, *129*, 7611 – 7619.
[9] J. Razkin, H. Nilsson, L. Baltzer, *J. Am. Chem. Soc.* **2007**, *129*, 14752 – 14758; J. Razkin, J. Lindgren, H. Nilsson, L. Baltzer, *ChemBioChem* **2008**, *9*, 1975 – 1984.
[10] J. F. Riordan, K. D. McElvany, C. L. Borders, Jr., *Science* **1977**, *195*, 884 – 886.
[11] X. Lopez, M. Schaefer, A. Dejaegere, M. Karplus, *J. Am. Chem. Soc.* **2002**, *124*, 5010 – 5018.
[12] T. Kato, T. Takeuchi, I. Karube, *Chem. Commun.* **1996**, 953 – 954; C. Gnaccarini, S. Peter, U. Scheffer, S. Vonhoff, S. Klussmann, M. W. Göbel, *J. Am. Chem. Soc.* **2006**, *128*, 8063 – 8067; U. Scheffer, A. Strick, V. Ludwig, S. Peter, E. Kalden, M. W. Göbel, *J. Am. Chem. Soc.* **2005**, *127*, 2211 – 2217.
[13] C. Schmuck, *Chem. Eur. J.* **2000**, *6*, 709 – 718; V. Bickert, PhD Thesis, Wuerzburg, **2008**.
[14] C. Schmuck, *Coord. Chem. Rev.* **2006**, *250*, 3053 – 3067; C. Schmuck, P. Wich, *Top. Curr. Chem.* **2007**, *277*, 3 – 30.
[15] C. Schmuck, V. Bickert, M. Merschky, L. Geiger, D. Rupprecht, J. Dudaczek, P. Wich, T. Rehm, U. Machon, *Eur. J. Org. Chem.* **2008**, 324 – 329.
[16] The synthesis of the peptides will be reported elsewhere.
[17] On the basis of a combinatorial approach, we recently identified octapeptides containing both histidine and guandiniocarbonyl pyrrole residues as efficient catalysts for phosphate hydrolysis: C. Schmuck, J. Dudaczek, *Org. Lett.* **2007**, *9*, 5389 – 5392.

