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Research Paper

A bridge between the RNA and protein worlds? Accelerating delivery of chemical reactivity to RNA and DNA by a specific short peptide $(AAKK)_4$

Robert C. Bergstrom, Lynn D. Mayfield, David R. Corey*

Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9041, USA

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Abstract

Background: RNA can catalyze diverse chemical reactions, leading to the hypothesis that an RNA world existed early in evolution. Today, however, catalysis by naturally occurring RNAs is rare and most chemical transformations within cells require proteins. This has led to interest in the design of small peptides capable of catalyzing chemical transformations.

Results: We demonstrate that a short lysine-rich peptide $(AAKK)_4$ can deliver a nucleophile to DNA or RNA and amplify the rate of chemical modification by up to 3400-fold. We also tested similar peptides that contain ornithine or arginine in place of lysine, peptides with altered stereochemistry or orientation, and peptides containing eight lysines but with different spacing. Surprisingly, these similar peptides function much less well, suggesting that specific combinations of amino acids, charge distribution, and stereochemistry are necessary for the rate enhancement by $(AAKK)_4$.

Conclusions: By appending other reactive groups to $(AAKK)_4$ it should be possible to greatly expand the potential for small peptides to directly catalyze modification of DNA or RNA or to act as cofactors to promote ribozyme catalysis. © 2001 Published by Elsevier Science Ltd.

Keywords: $(AAKK)_4$; Lysine-rich peptide; Nucleophile delivery; Ribozyme catalysis; RNA world

1. Introduction

RNA can catalyze many chemical reactions, supporting the hypothesis that an RNA world once existed [1–4]. Today, however, most chemical transformations within cells require proteins. This in turn has led to interest in the design of small peptides capable of catalyzing chemical transformations [5–7]. The most striking of these small designed peptides were cyclic mimics of chymotrypsin and trypsin which were reported to efficiently carry out both ester and amide hydrolysis [8]. Unfortunately, this work could not be reproduced by other laboratories [9,10]. Two factors contributed to the inability to readily observe catalysis by peptide mimics of trypsin and chymotrypsin; (i) no means for binding of substrate were present, and (ii) peptide bond hydrolysis is an energetically demanding reaction that requires precise alignment of multiple catalytic residues. We reasoned that the lack of reproducible catalysis by protease mimics should not be taken as an indication that small peptides cannot dramatically accelerate chemical reactivity and that the criteria for designing a first generation of functional small peptides should be simplified.

To generate simple peptides that can bind and chemically transform a nucleic acid substrate, we took advantage of a previous observation from our laboratory that a cationic peptide modeled after the surface of staphylococcal nuclease can enhance the hybridization of attached oligonucleotides to complementary DNA sequences. This peptide $(AAKK)_4$ increased the rate constant for association between an oligonucleotide–peptide conjugate and a duplex DNA target, k_a, by as much as 48 000-fold relative to k_a for hybridization of an unmodified oligonucleotide [11]. Presumably, the mechanism of this acceleration involves electrostatic interactions between the peptide and the repetitive anionic phosphodiester backbone of DNA. Their ability to accelerate hybridization suggested that cat-

* Correspondence: David R. Corey; E-mail: corey@chop.swmed.edu

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ionic peptides might also enhance the rate of delivery of chemically reactive functional groups. If true, this enhancement would facilitate the transition from peptides being passive agents capable of assisting nucleic acid recognition to peptides being active agents that more closely resemble enzymes.

Here we describe use of the short lysine-rich peptide $(AAKK)_4$ to accelerate the rate of DNA or RNA modification by up to 3400-fold. Surprisingly, other closely related peptides that contain the same number of positively charged residues function much less efficiently. These results suggest that peptide $(AAKK)_4$ has unusually favorable properties that make it a simplified starting point for the evolution or design of small peptide catalysts.

2. Results and discussion

To test the hypothesis that cationic peptides would accelerate the delivery of attached functional groups to DNA we chose disulfide exchange as a model reaction (Fig. 1). We included a cysteine at the N-terminus of the cationic peptide to serve as a nucleophile, and introduced a thiopyridyl activated thiol onto the 5′-terminus of single-strand DNA, single-stranded RNA, or hairpin DNA oligonucleotides to act as a reactive target. This scheme was advantageous for testing our hypothesis because the disulfide exchange reaction is facile and because thiopyridyl release is easily and unambiguously observed at 342 nm [12]. While the testing of protease mimics has been handicapped by the difficulty of amide bond hydrolysis and the potential for misleading background reactivity, our experiments allow the basic features of the recognition of substrate by reactive peptides to be conveniently monitored and readily characterized. Furthermore, our focus on interactions between peptides and nucleic acids targets the evolutionary junction between the RNA and protein worlds where small peptide enzymes would have been necessary. Knowledge of these interactions is important for modeling the mechanism of early protein evolution, designing peptides with increasingly sophisticated catalytic function, and for guiding experiments aimed at co-evolving increasingly active complexes of peptides and RNA and DNA.

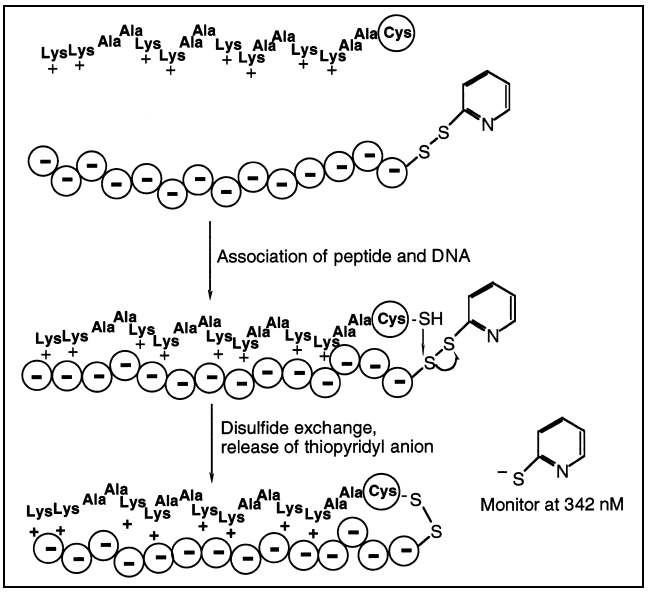

Fig. 1. Schematic describing association of cationic peptide with DNA and subsequent disulfide exchange and release of thiopyridyl anion. While this schematic shows only one binding orientation, it is likely that several productive binding orientations are possible.

Table 1
Values for k_{obs} characterizing the reaction of peptides with *S*-thiopyridyl-labeled DNA or RNA oligomers

Peptide	Sequence	k_{obs} (M^{-1} s^{-1})		
		ssDNA	dsDNA	ssRNA
	varied numbers of lysines			
I	CAAKKAAKKAAKKAAKK	34 000	110	18 000
II	CAAKKAAKKAAKK	800	nd	1 700
III	CAAKK	70	40	nd
	varied overall charge			
IV	CAADKAADKAADKAADK	100	nd	nd
V	CAASKAASSAASSAASK	150	230	390
	varied arrangement of lysines			
VI	CAAAAKKKKAAAAKKKK	330	70	nd
VII	CAAAKAAAKAAAKAAAK	660	150	450
VIII	CAKAKAKAKAKAKAKAK	540	750	180
IX	KKAAKKAAKKAAKKAAC	480	370	200
X	D-CAAKKAAKKAAKKAAKK	990	20	2 400
	varied identity of paired charge residue			
XI	CAARRAARRAARRAARR	140	10	190
XII	CAARKAARKAARKAARK	1 300	22	1 600
XIII	CAAOOAAOOAAOOAAOO	210	65	270

nd: not detectable above background. Peptide **X** contained D-amino acids throughout. All peptides are listed N- to C-termini. O = ornithine. All values represent an average of triplicate determinations. ds = double-stranded hairpin. ss = single-stranded.

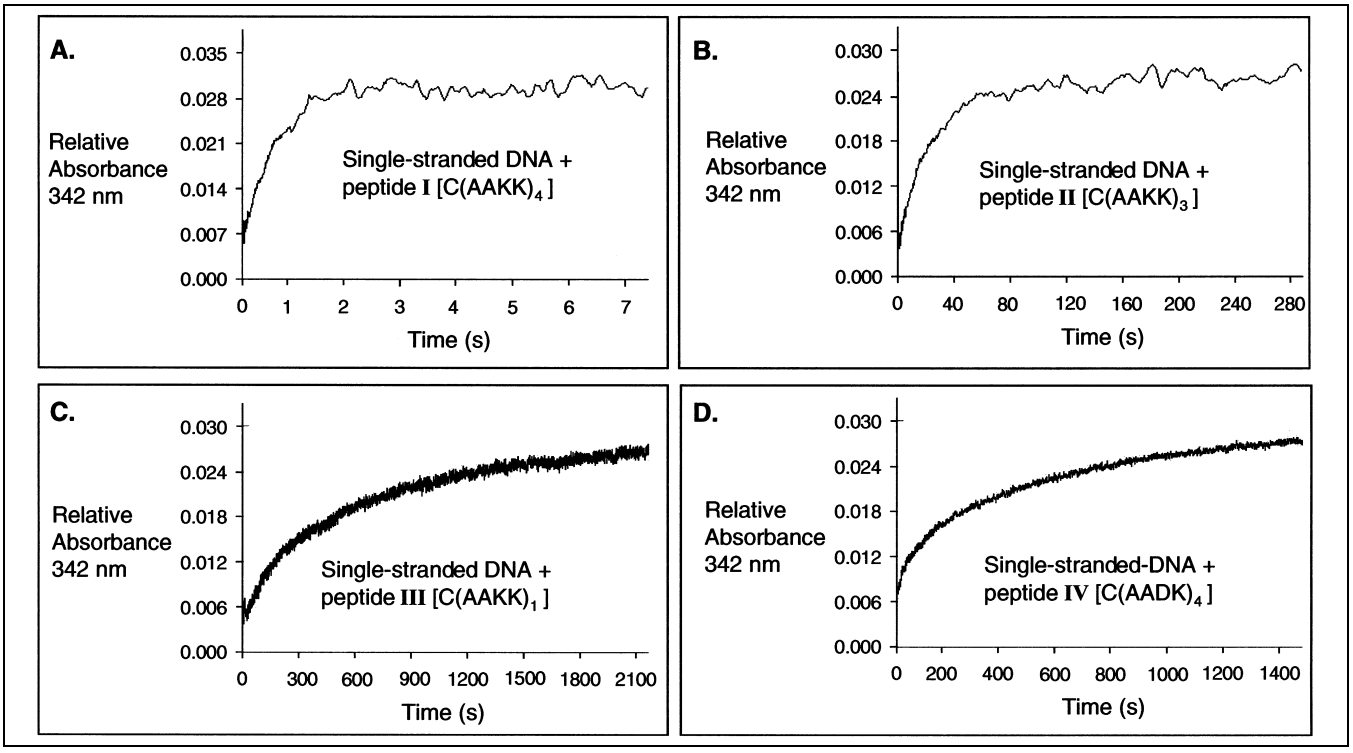

Fig. 2. Stopped flow spectrophotometry monitoring the release of thiopyridyl anion upon mixing 5′-*S*-thiopyridyl DNA with peptides that contain varying amounts of positive charge. (A) Peptide **I**. (B) Peptide **II**. (C) Peptide **III**. (D) Peptide **IV**. Oligonucleotide and peptide were present at a concentration of 5 μM.

2.1. Crosslinking of peptides with single-stranded DNA

The peptides used in these experiments varied in overall charge from 0 to +8 (Table 1). We observed rapid disulfide exchange upon addition of peptide **I**, [C(AAKK)$_4$], to a 5′-*S*-thiopyridyl DNA oligonucleotide containing the sequence 5′-TTCACCTAGATCCT-3′. This crosslinking was too fast to be measured by manual methods on a standard UV spectrophotometer and the initial rate of thiopyridyl release was monitored using stopped flow spectrophotometry. These data indicate that the reaction occurs with a second order rate constant, k_{obs}, of 34 000 M^{-1} s^{-1} (Table 1) (Fig. 2A). When the concentration of reactants was increased, the measured k_{obs} increased proportionally (data not shown), supporting the conclusion that the reaction is a second order process. k_{obs} for release of thiopyridyl upon addition of dithiothreitol (DTT) was 10 M^{-1} s^{-1}, indicating that the presence of the positively charged peptide is having a dramatic effect on disulfide exchange. A similar enhancement in disulfide exchange was observed when 5′-*S*-thiopyridyl-labeled DNA oligonucleotide 5′-CTCATATATACTTTAGAT-3′ was mixed with peptide **I**, indicating that the accelerated delivery of chemical reactivity that we observe is independent of DNA sequence.

We then tested peptides **II–V** to correlate the charge of the peptide with enhanced reactivity and found that the observed rate acceleration was dependent on the overall charge. Peptides **II** (six lysines) and **III** (two lysines), like peptide **I**, had paired lysines adjacent to paired alanines but exhibited much reduced k_{obs} values of 800 and 70 M^{-1} s^{-1}, respectively (Fig. 2B,C). Peptide **IV**, which has four lysines and a net overall charge of 0 exhibited a k_{obs} of 100 M^{-1} s^{-1} (Table 1, Fig. 2D), a reduction of 3400-fold relative to peptide **I**. We also assayed peptide **V**, which possessed only two lysines but also contained six serine residues. Similar to peptide **I**, peptide **V** contained eight residues capable of forming hydrogen bonds but this potential for hydrogen bonding yielded a k_{obs} of only 150 M^{-1} s^{-1}. Addition of magnesium chloride to a final concentration of 50 mM reduced k_{obs} for crosslinking by **I** to 400 M^{-1} s^{-1}, a decrease of 85-fold. Taken together, these results support the conclusion that enhanced reactivity is dependent upon electrostatic interactions between lysine and the DNA backbone.

We chose to incorporate paired lysines into our initial peptide designs because they are a common motif on the surface of staphylococcal nuclease, a well studied protein that binds DNA. We had reasoned that this motif acts to promote association with nucleic acids. To determine whether the pairing of lysines contributes to the accelerated reactivity we observe, we tested peptides containing different arrangements of lysine residues relative to each other and relative to the peptide as a whole. We compared peptide **VI**, which contained eight lysines arranged in two sets of four, peptide **VII**, which contained four single ly-

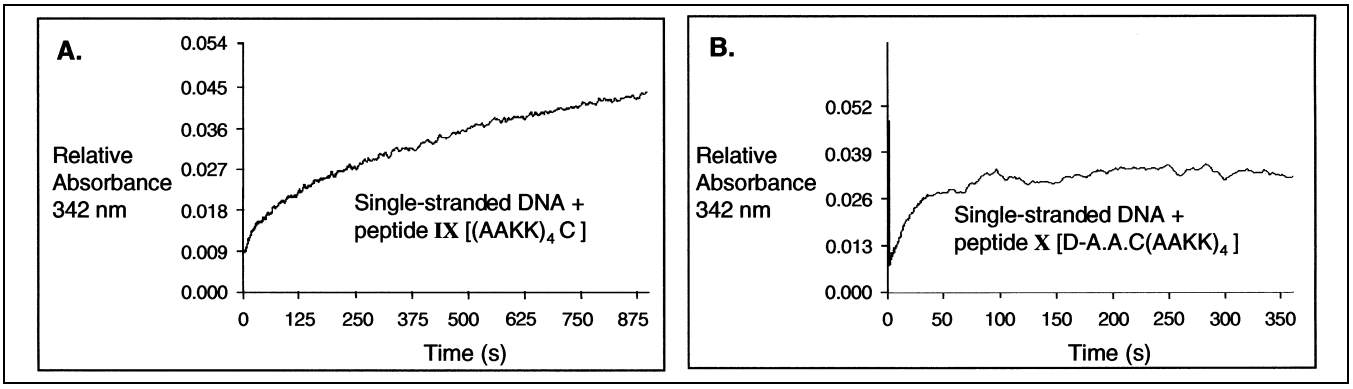

Fig. 3. Stopped flow spectrophotometry monitoring the release of thiopyridyl anion upon mixing 5′-*S*-thiopyridyl anion with peptides that contain a different orientation or stereochemistry for positive charge. (A) Peptide **IX**, a peptide with N- and C-termini reversed relative to peptide **I**. (B) Peptide **X**, a peptide analogous to **I** but containing D-amino acids. Oligonucleotide and peptide were present at a concentration of 5 μM.

sines, each separated by three alanines, and peptide **VIII** with eight single lysines, each separated by a single alanine. We found that k_{obs} values for peptide **VI–VIII** were reduced 50–100-fold relative to **I**. This substantial differential is observed even though peptides **VI** and **VIII** contain the same overall charge and number of lysines as found in **I**. One conclusion is that the pairing of two lysine residues in tandem has advantages for promoting nucleic acid association.

2.2. *Effect of peptide orientation and stereochemistry*

To further investigate the origin of enhanced recognition by peptide **I**, we examined the importance of the spatial orientation and stereochemistry of lysine residues. We had expected that these would not be important variables because of the flexibility of the DNA and peptide chains and because of the simplicity of our model reaction. Surprisingly, however, we found that peptide **IX**, which had its N- and C-termini reversed relative to **I**, possessed a k_{obs} for crosslinking of only 480 M^{-1} s^{-1} (Fig. 3A), a 70-fold reduction. Similarly, peptide **X** that was analogous in sequence to **I** but contained D- rather than L-amino acids reacted with a k_{obs} of 990 M^{-1} s^{-1} (Fig. 3B), a 35-fold reduction relative to **I**. The reduced k_{obs} values for crosslinking by **IX** and **X** indicate that the configuration of charged residues and their orientation relative to the nucleophile are important determinants for the enhanced reactivity exhibited by **I**. Given the importance of spatial orientation and stereochemistry in this simple system, they are likely to be even more critical considerations for future work aimed at the design of more elaborate catalysts.

2.3. *Effect of substituting ornithine or arginine in place of lysine*

As noted above, we chose lysine as a charged residue because it dominates the surface of staphylococcal nuclease (there are five lysine pairs and 11 single lysines throughout the 149 amino acid protein). To determine the effect of replacing lysine by other charged residues, we obtained peptides **XI–XIII** containing eight arginines, a combination of eight arginines or lysines, and eight or-

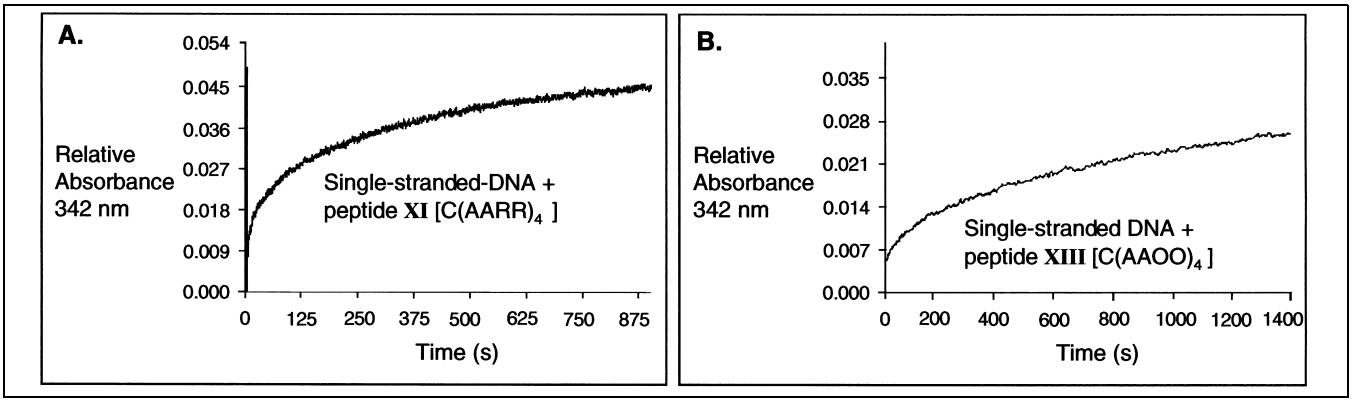

Fig. 4. Stopped flow spectrophotometry monitoring the release of thiopyridyl anion upon addition of peptides containing eight arginines or ornithines. (A) Peptide **XI**, a peptide containing eight arginines. (B) Peptide **XIII**, a peptide containing eight ornithines. Oligonucleotide and peptide were present at a concentration of 5 μM.

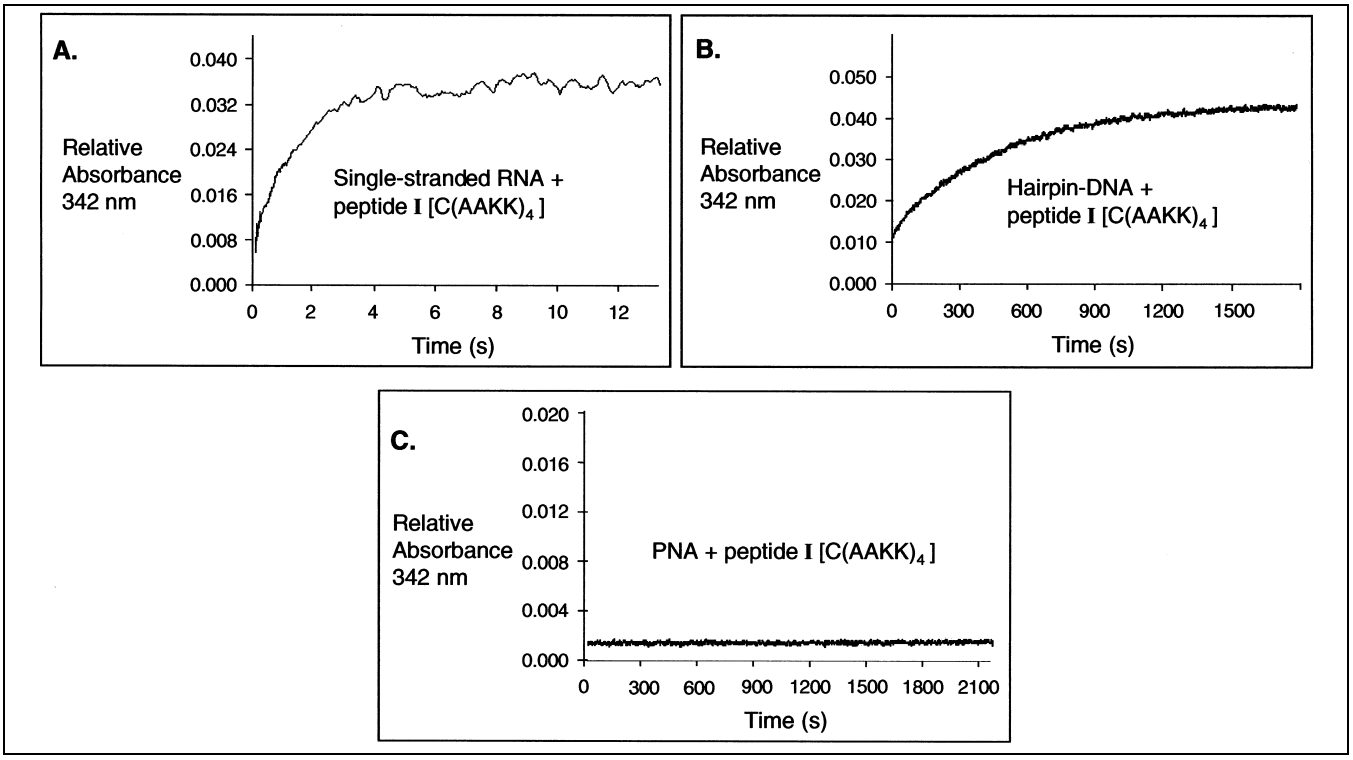

Fig. 5. Stopped flow spectrophotometry monitoring the release of thiopyridyl anion upon addition of peptide **I** to *S*-thiopyridyl-labeled duplex DNA, single-stranded RNA, and single-stranded PNA. (A) Single-stranded RNA. (B) Duplex DNA. (C) PNA. Oligonucleotide or PNA and peptide were present at a concentration of 5 μM.

nithines, respectively (Table 1, Fig. 4A–C). The reaction pH was 8.0, so the overall charge was +8 regardless of the pK_a of the charged amino acid. We found that peptides **XI–XIII** reacted with *S*-thiopyridyl DNA with k_{obs} values of 140, 1300, and 210 M^{-1} s^{-1}, respectively, values reduced 30–240-fold relative to the k_{obs} value for crosslinking by **I**. The preference for lysine over other charged residues demonstrates the identity of the charged amino acid is as important for efficient crosslinking as the quantity and placement of charge. It is particularly striking that lysine is highly preferred over ornithine, an analog that lacks one methylene group. This finding reinforces the notion that the accelerated delivery of reactivity that we observe is not a general property of charged peptides but is restricted to a small subset of peptides, and that peptide **I** has specific advantages for the evolution of catalysts or for their design.

2.4. Crosslinking of peptides to single-stranded RNA, hairpin DNA, and peptide nucleic acid (PNA) oligomers

To begin to generalize our results to other nucleic acids we examined the reaction of peptides **I–XIII** with single-stranded RNA and self-complementary hairpin DNA oligonucleotides. The RNA was analogous in sequence to the single-stranded DNA we had previously tested, as was one strand of the hairpin DNA. We found that crosslinking to RNA occurred with k_{obs} values that were similar to those observed for crosslinking to single-stranded DNA (Fig. 5A). Thus it appears that the delivery of chemically reactive groups can tolerate changes in the nature of the nucleic acid substrate better than changes to the sequence and charge of the peptide. By contrast to the similarity of single-stranded DNA and RNA, k_{obs} values for crosslinking to duplex hairpin DNA were low for every peptide tested (Fig. 5B). The simplest explanation for this lack of reactivity is that the extra strand blocks access to the thiopyridyl disulfide, but it is also possible that peptides were less able to rapidly associate with duplex DNA.

As a final confirmation of the importance of electrostatic interactions between peptides and the phosphate backbone we assayed crosslinking of **I** to a cysteine containing PNA oligomer (Fig. 5C) [13]. PNAs are a neutral DNA/RNA mimic in which the phosphate deoxyribose backbone has been replaced by uncharged *N*-(2-aminoethyl)glycine linkages, and PNAs hybridize to complementary sequences according to rules that are very similar to those governing binding of DNA or RNA. Use of PNAs allows us to examine the importance of electrostatic interactions while maintaining oligomer target sequence and reaction conditions constant. We found that k_{obs} for crosslinking of the PNA was < 20 M^{-1} s^{-1} (Fig. 5C), further supporting the conclusion that electrostatic interactions are necessary for the enhanced crosslinking we observe.

3. Significance

The fact that a cationic polymer can associate with DNA and RNA is not surprising given the lengthy literature on the association of cationic molecules with nucleic acids [14,15]. What is surprising is that $(AAKK)_4$ is so much more efficient than peptides with similar charge, suggesting that the enhanced ability to deliver reactivity is not a general property possessed by any positively charged polymer, rather it requires a precise combination of stereochemistry, charge distribution, and amino acid chemistry.

We chose disulfide exchange as a model reaction to simplify evaluation of reactivity and avoid artifactual results that have been encountered in earlier efforts to design reactive peptides. It is not difficult, however, to imagine the delivery of many other types of chemically reactive groups using similar peptides. Some chemically reactive moieties have already been shown to promote RNA cleavage, including metal complexes [16–21] and nucleophiles such as imidazole [22,23] and amines [24–27].

Perhaps more relevant to the evolution of RNA catalysis, enhanced delivery of functional groups by peptides should enhance ribozyme catalysis by facilitating the use of amino acid cofactors [28,29]. Once a peptide begins to contribute to RNA catalysis, one can envision steady evolution of a replacement reaction mechanism that progressively transforms the peptide from a cofactor into a species that more closely resembles an enzyme.

4. Materials and methods

4.1. Synthesis of 5′-thiopyridyl oligonucleotides, modified PNAs, and peptides

5′-*S*-Trityl RNA and DNA oligonucleotides were synthesized by standard methods using *S*-trityl-6-mercaptohexyl-2-cyanoethyl-*N*,*N*-diisopropyl-phosphoroamidite (C-6 thiol modifier) (Clontech, Palo Alto, CA, USA) or were purchased from the HHMI Biopolymer/Keck Foundation of Biotechnology Resource Laboratory (New Haven, CT, USA). DNA oligonucleotides used in these experiments were 5′-*S*-trityl-CTCATATATACTTTAGAT-3′, 5′-*S*-trityl-TTCACCTAGATCCT-3′ and 5′-*S*-trityl-TTCACCTAGATCCTAGGATCTAGGTGAA-3′ (which forms a hairpin). The RNA oligonucleotide used in these experiments was 5′-*S*-trityl-UUCACCUAGAUCCU-3′ and was obtained from Oligos Etc. (Wilsonville, OR, USA).

The 5′-thiopyridyl group was added after resuspension of the oligonucleotide in 0.1 M triethylamine acetate buffer, pH 6.5. First, the *S*-trityl group was removed from the oligonucleotide by addition of silver nitrate to a final concentration of 150 mM and incubated at room temperature for 30 min. The thiol was maintained in a reduced state and silver was precipitated by addition of DTT to a final concentration of 150 mM with subsequent centrifugation at 12 000 rpm in a desktop microcentrifuge at room temperature for 15 min. The resulting supernatant was washed three times with ethyl acetate to remove DTT. 2′,2′-Dithiodipyridine (Aldrich, Milwaukee, WI, USA) was added to a final concentration of 5 mg/ml for 30 min at room temperature. After this incubation, the supernatant was washed six times with ether to remove dithiodipyridine, frozen, and lyophilized. The modified oligonucleotide was resuspended in TE buffer and desalted using Bio-Rad (Hercules, CA, USA) Spin-6 chromatography columns. The yield of modified oligonucleotide was determined by complete reduction of a sample with 10 mM DTT and measurement of absorbance at 342 nm.

The PNA oligomer used in these experiments was NH_2-Cys-TTCACCTAGATCCT-3′-COOH and was synthesized on an Applied Biosystems (Foster City, CA, USA) Expedite 8909 Synthesizer with Fmoc chemistry using monomer reagents from Applied Biosystems [30]. The cysteine containing PNA was treated with 2,2′-dithiodipyridine and the thiopyridyl-labeled PNA was purified as described [31]. Peptides were synthesized using solid-phase synthesis on an Applied Biosystems model 430A peptide synthesizer with Fmoc chemistry. PNAs and peptides were purified using reverse-phase high performance liquid chromatography and their molecular weights confirmed using time-of-flight mass spectrometry (MALDI-TOF). All peptides had free amino-termini and amidated C-termini.

When peptide, DTT, and oligonucleotide are mixed together, specific reaction of peptide and oligonucleotide was confirmed by monitoring the formation of oligonucleotide–peptide conjugate by Mono Q anion exchange FPLC (Pharmacia, Peapack, NJ, USA) as described [32]. Crosslinked oligonucleotide–peptide conjugate had a significantly lower retention time than unmodified oligonucleotide. DTT was present in stock solutions of peptide at a concentration of 5 mM to maintain the peptides in a reduced state. The final diluted reaction concentrations of 10 μM DTT did not significantly contribute to the measured rate of thiopyridyl release. Aqueous DTT stock solutions were prepared from solid immediately prior to use. All reactants were stored in 10 mM Tris–Cl pH 8.0 buffer.

4.2. Measurement of rate constants for delivery of reactive nucleophile and data analysis

The ability of cysteine containing peptides to accelerate release of thiopyridyl anion from labeled oligonucleotides was measured by monitoring thiopyridyl release at 342 nm. There is a tendency for complexes between oligonucleotides and cationic peptides to precipitate causing the measured absorbance to increase throughout the visible range. To confirm that this is not happening, the maxima at 342 nm should be clearly visible and the baseline reader at 400 nm should be 0. Relatively slow reactions could be monitored by manually mixing the components and following the crosslinking using a standard Hewlett Packard 8542 diode array UV spectrophotometer. *S*-Thiopyridyl oligonucleotide and reaction buffer were added to the cuvette and the reaction was initiated with addition of peptide. The increase in absorbance at 342 nm was measured over time. Reactions were carried out at

25°C with 10 mM Tris–Cl pH 8.0, 5 μM of modified oligonucleotide, and 5 μM peptide.

Both slow and rapid reactions were also measured using a Bio-logic/Molecular Kinetics (Claix, France/Pullman, WA, USA) stopped flow spectrophotometry apparatus. The pumps and the stopped flowed module were from Bio-Logic/Molecular Kinetics. The buffer and the modified oligonucleotide being tested were present in one injector of the apparatus. The peptide was present in the other injector. Mixing occurred with a lag time of approximately 80 ms allowing accurate measurement of the fastest reaction with peptide **I**. All reactions were run until no further increase in absorbance was observed. Data were analyzed using Bio-Kine for Windows version 2.04 (Bio-Logic, Claix, France) and Sigma Plot for Windows (SPSS Science, Chicago, IL, USA). No change in absorbance after addition of 2 mM DTT at the end of each reaction confirmed that reactions were complete. Initial reaction rates were determined using a molar absorption coefficient $\varepsilon = 7060$ for the thiopyridyl group.

Acknowledgements

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