48000-fold Acceleration of Hybridization by Chemically Modified Oligonucleotides

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Most studies on the hybridization of chemically modified oligonucleotides have focused on the strength of binding as measured by the melting temperature, T_m.[1] Equally important, however, is the rate constant for association, k_a, between oligomer and complement,[2] as k_a dictates the efficiency of hybridization within a given period. Acceleration of this rate would be especially beneficial for targeting DNA sequences that are rare or that exist within base-paired structures in which the single-stranded regions required to initiate hybridization are only transiently accessible.

We have previously demonstrated that covalent attachment of staphylococcal nuclease to an oligonucleotide generates an adduct capable of hybridizing to a complementary target within duplex DNA with a k_a 12000-fold greater than that possessed by the analogous unmodified oligonucleotide.[3] It is likely that the enhanced k_a is due to interactions between the lysine-rich positively charged surface of staphylococcal nuclease and the negatively charged phosphodiester backbone of the DNA template. This interaction would accelerate specific hybridization by increasing the effective concentration of oligomer near the target sequence.[4]

While the observed acceleration of hybridization in these earlier studies was dramatic, attachment of a protein limits the potential for *in vivo* application as protein-linked conjugates would probably not be able to cross cellular membranes. The utility of accelerated hybridization, therefore, would be greatly extended if the protein could be replaced by positively charged peptides. It was not obvious, however, that short peptides, which lack stable structure, would facilitate hybridization as effectively as staphylococcal nuclease, a rigid polypeptide that possesses a three-dimensional structure whose native function is to associate with DNA. Therefore, to determine if hybridization could be accelerated by conjugation of smaller molecules, we examined the rate of hybridization of oligonucleotide–peptide adducts.

The optimization of oligonucleotide hybridization, cellular permeability, and cellular localization through the conjugation of oligonucleotides to peptides has been the subject of numerous biophysical and synthetic investigations.[5] We have synthesized oligonucleotide–peptide adducts by disulfide exchange between cysteine and a 5′-*S*-thiopyridyl activated thiol on an oligonucleotide.[6] Hybridization of the adducts to a target sequence within supercoiled duplex DNA that contains an inverted repeat was monitored by their ability to act as primers for dideoxy sequencing with DNA polymerase (Figure 1a).[3,7] This assay is advantageous, as polymerization yields DNA sequence information that unambiguously confirms the site of annealing. Additionally, quantitation of the elongation products allows estimation of the velocity of hybridization and k_a.[3] An inverted repeat was chosen as a target site because such sequences are known to undergo transient strand separation, thus providing a single-stranded region as a nucleation site for strand invasion. Other DNA motifs that are thought to have a propensity to become single stranded include triplet repeats, duplex unwinding elements, H-form DNA, Z-DNA, matrix attachment regions, and RNA polymerase open complexes.[8]

The oligonucleotide–peptide adducts primed DNA polymerization, and polymerization increased proportionally with the time allowed for annealing to duplex template (Figure 1b,c). Attachment of staphylococcal nuclease increased k_a for hybridization by 11400-fold relative to hybridization by the analogous unmodified oligonucleotide, a ratio similar to that reported previously[3] (Table 1). Attachment of peptides also yielded enhanced association constants, and accelerated hybridization varied with the quantity and placement of positive charge. Peptides containing sequences found in histones[9] (**III**) or the DNA-binding domain of HMG-I protein[10] (**IV**), which were known to bind independently to DNA, accelerated hybridization by 7- and 11400-fold, respectively. Peptides containing four (**V**), six (**VI**), or eight (**VIII**) lysines increase k_a by 5-, 67-, or 48000-fold[11] (Figure 1b,c). Paired lysines were chosen for these peptides, because five lysine pairs occur on the surface of staphylococcal nuclease.[12] A peptide (**VII**), which was coupled to the oligonucleotide via an internal cysteine and which possessed eight lysines, enhanced k_a 910-fold, significantly less than **VIII**, suggesting that the effect of positive charge is greater

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(6) An oligonucleotide was synthesized by standard methods using C-6 Thiolmodifier (Clonetech).[3] The oligonucleotide sequence was 5′-GGATCT-TCACCTAGATCCT-3′, which corresponds to bases 1546–1565 in pUC19. Attachment of the 5′-*S*-thiopyridyl oligonucleotide to peptides allowed the adducts to be separated from unreacted oligonucleotide by Mono Q anion exhange chromatography (Pharmacia).

(7) To initiate annealing, oligonucleotide or oligonucleotide–peptide adducts were added to a solution of plasmid in 10 mM Tris-Cl, pH 8.0, at 37 °C. Annealing was terminated by chilling on ice and elongation reactions as described.[3] Oligonucleotide adducts were directed to a DNA sequence containing an inverted repeat sequence within supercoiled pUC19 that has been shown to efficiently incorporate oligonucleotides and oligonucleotide adducts. Corey, D. R.; Pei, D.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 8523–8525. Corey, D. R.; Munoz-Medellin, D.; Huang, A. *Bioconjugate Chem.* **1995**, *9*, 93–100. pUC19 DNA was purified by a mild lysis procedure to ensure the absence of contaminating denatured DNA. Murchie, A. I. H.; Lilley, D. M. *Methods Enzymol.* **1992**, *211*, 158–180.

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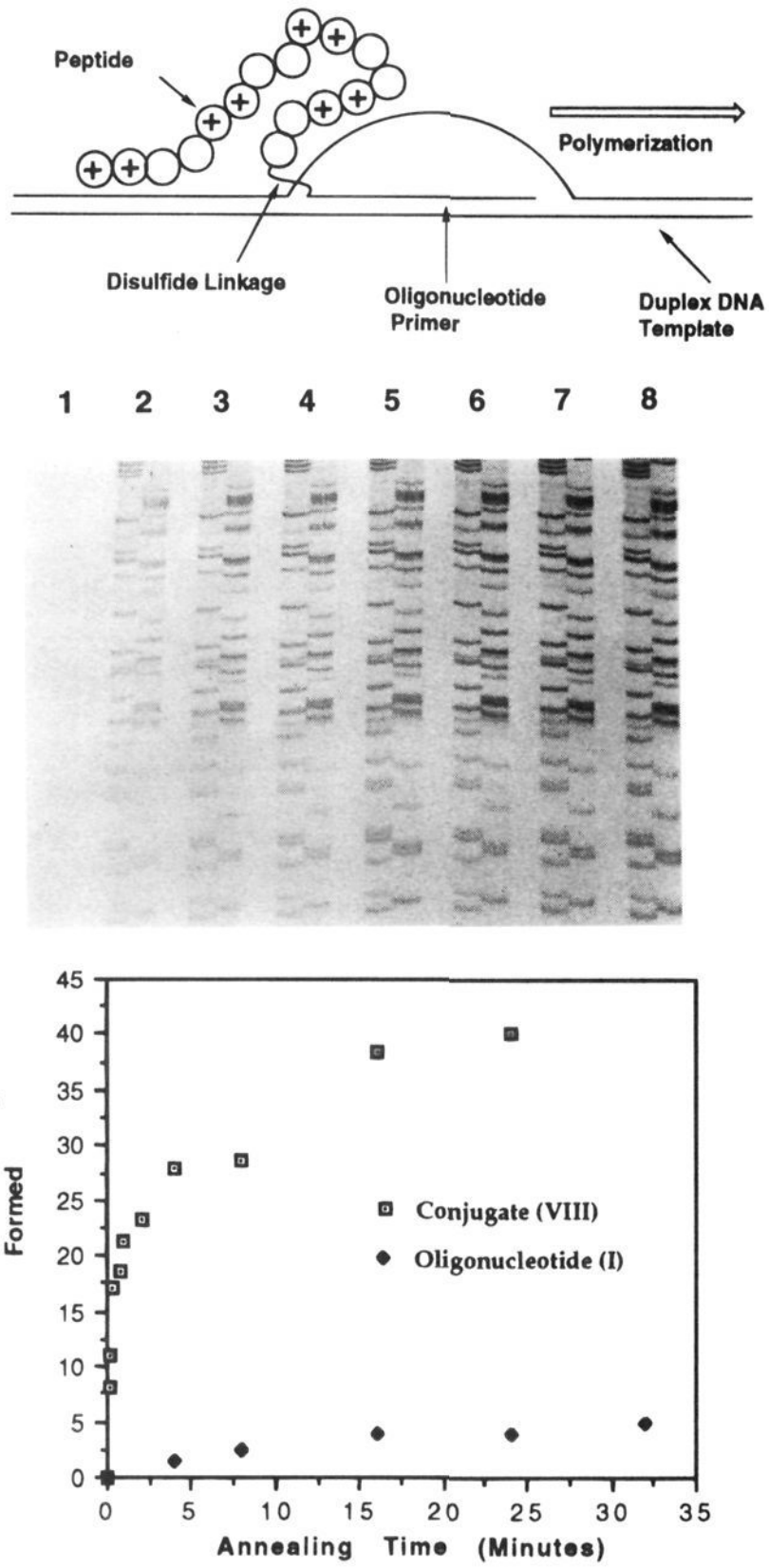

Figure 1. (a) (top) Schematic demonstrating a possible mode of annealing an oligonucleotide–peptide conjugate to duplex DNA. (b) (middle) Effect of annealing time on DNA sequencing by oligonucleotide–peptide adduct **VIII**. Only dideoxyadenosine and dideoxycytidine termination reactions are shown. Annealing mixtures contained 0.01 μM pUC19 and 0.06 μM oligonucleotide–peptide adduct **VIII**. Lanes 1–8, 0, 0.15, 0.45, 1.5, 4, 8, 16, and 25 min annealing times. Solutions containing DNA were maintained at 37 °C, and primer was added to commence annealing. At specified time points aliquots were withdrawn and chilled in an ice/water bath to terminate annealing. Stop buffer for elongations contained 5 mM dithiothreitol to release peptide prior to electrophoresis. (c) (bottom) Phosphorimager quantitation of elongation products after priming by oligonucleotide **I** and oligonucleotide–peptide conjugate **VIII** as a function of time. Oligonucleotide **I** was present at 2.5 μM and was annealed to 0.02 μM plasmid at 37 °C. The percentage of plasmid to incorporate oligonucleotide was estimated by visualization of the relative amounts of unmodified plasmid and the elongation product–plasmid complex after separation by agarose gel electrophoresis. As short elongation products are likely to dissociate during electrophoresis, the percentage represents a lower limit.

Table 1. Effect of 5′-Modifications on Association Constants of Oligonucleotide Adducts for Duplex DNA

	5′ modification	k_a (M^{-1} s^{-1})	k_{rel}
(**I**)	no modification	11	1
(**II**)	SNase[a]	125 000	11 400
(**III**)	CGGSPRKSPRK	77	7
(**IV**)	CTPKRPRGRPKK	125 000	11 400
(**V**)	CAAGAKKAAGAKK	55	5
(**VI**)	CAAKKAAKKAAKK	740	67
(**VII**)	KKAAKKACAKKAAKK	10 000	910
(**VIII**)	CAAKKAAKKAAKKAAKK	530 000	48 000
(**IX**)	CAARRAARRAARR	760	69
(**X**)	CAARRAARRAARRAARR	1800	164

[a] SNase: staphylococcal nuclease.

when displayed from a single extended chain. Attachment of arginine-containing peptides **IX** and **X** also yielded increases in k_a, although the incremental change from six to eight arginines was less than that observed for lysine-containing adducts **VI** and **VIII**.

One of the obstacles to the hybridization of peptide–oligonucleotide conjugates or other oligomers to transiently single-stranded regions within duplex DNA is that, *in vitro*, hybridization under presumed "physiologic" conditions of ionic strength stabilizes B-form DNA structure and prevents the unpairing necessary to initiate base-pairing. Indeed, while hybridization by conjugate **VIII** did occur in the presence of 70 mM NaCl or 70 mM potassium glutamate, the presence of greater than 0.5 mM $MgCl_2$ effectively prevented strand invasion. However, because of the complex and crowded nature of the intracellular space, the effective ionic strength *in vivo* is unclear. For example, in the nucleus, concentrations of DNA, RNA, and protein are estimated at 30, 100, and 250 mg/mL, respectively, far above the concentrations employed in *in vitro* experiments.[13] The high concentration of DNA found in the nucleus probably titrates much of the available magnesium, creating conditions of low effective ionic strength. Moreover, there is strong evidence that transiently single-stranded regions within duplex DNA are common *in vivo*,[8] making them intrinsically accessible targets for hybridization.

Accelerated hybridization of peptide–oligonucleotide conjugates to DNA demonstrates that the rate of hybridization can be manipulated by relatively simple chemical modifications. Surprisingly, incremental increases in charge appear to yield substantial increases in k_a. The observed rate enhancements do not require that amino acids be displayed in a constrained conformation; rather the presence of positive charge is sufficient to accelerate hybridization even if borne by a flexible chain. Positively charged peptides also facilitate membrane association and cellular uptake,[14] and may therefore prove doubly advantageous for *in vivo* targeting. Non-peptidic positively charged chemical motifs should also accelerate hybridization, and the development of optimal adducts presents a challenging problem at the interface of synthetic chemistry and biomolecular design.

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