

Hybrid Enzymes and the Sequence-Specific Cleavage of Nucleic Acids

David R. Corey, Ronald N. Zuckermann, Peter G. Schultz
University of California, Berkeley, Berkeley CA, 94720, USA

Catalysts have been generated that sequence-specifically hydrolyze RNA and DNA at predefined sites, i.e., a new class of restriction enzymes. New binding domains (oligonucleotides or peptides of defined sequence) were introduced site-selectively into the relatively nonselective phosphodiesterases, staphylococcal nuclease and ribonuclease S, to generate hybrid enzymes that site-specifically cleave nucleic acids. Subsequent mutagenesis of the hybrid enzymes has generated enzymes that site-selectively hydrolyze one bond in large RNAs and DNAs in a catalytic fashion. We have been able to use these hybrid enzymes to selectively cleave duplex plasmid DNAs via D-loop formation. This work has not only resulted in powerful tools for studying RNA and DNA structure but also may provide a general strategy for designing other selective hybrid enzymes for important biological transformations, such as the selective cleavage of genomic DNA, or selective cleavage of peptide amide bonds.

1 Introduction	3
2 Semisynthetic Proteins	3
2.1 Thiol-subtilisin	4
2.2 Flavo-papain	4
2.3 Semisynthetic Antibodies	5
3 Sequence-Specific Hybrid Nucleases	6
3.1 Hybrid Nucleases as Tools for the Sequence-Selective Cleavage of RNA and DNA	7
3.2 Design Considerations	8
4 Oligonucleotide-Directed Staphylococcal Nuclease	9
4.1 Oligonucleotide Binding Site	9
4.2 Properties of Staphylococcal Nuclease	11
4.3 Use of Cysteine in Protein Engineering	12
4.4 Synthesis of the Hybrid Nuclease	13
4.5 Sequence-Selective Cleavage of Unstructured DNA Substrates	13
4.6 Optimization of the Hybrid Nuclease	15
4.7 Cleavage of Structured DNA Substrates	15
4.8 Catalytic Turnover by Hybrid Nucleases	17
4.9 Alteration of the Interdomain Linkage	18

4.10	Mechanisms for Specific versus Nonspecific Hydrolysis	19
4.11	Selective Hydrolysis of Duplex DNA by a Hybrid Nuclease	20
4.12	Sequence-Selective Cleavage of RNA	22
4.13	Sequence-Selective Cleavage of Structured RNA Substrates	23
4.14	Enzymatic Manipulation of the Fragments from Selective RNA Hydrolysis	25
5	Oligonucleotide-Directed RNase A	25
5.1	RNase S Hybrid Nucleases	27
5.2	RNase A Hybrid Nucleases	29
6	Conclusion	29
7	References	29

1 Introduction

Chemists and biologists are focusing considerable effort on the design and synthesis of novel catalysts with enzyme-like specificities. Chemists have become increasingly proficient in the synthesis of selective catalysts that complex and transform small molecules or structural motifs. Chiral transition metal complexes are proving to be useful general catalysts in organic synthesis [1, 2]. Cavity-containing hosts are also being derivatized with chemically reactive groups in an effort to construct catalysts that mimic and generalize enzyme-catalyzed reactions [3–5]. Although catalysts of this sort have not yet proven generally useful in synthesis, they do have potential for studying the mechanisms of molecular recognition and catalysis.

As the size and complexity of substrates increase, the ability to generate catalysts for rationally manipulating their structures becomes quite limited. The synthesis of such catalysts requires the generation of bifunctional molecules which combine binding sites capable of discriminating complex polyfunctional molecules, with the appropriate chemical groups to carry out subsequent catalysis. To date, only limited success has been achieved in the rational design of receptors capable of selectively binding complex molecules. Most efforts have focused on modification of the specificity of existing proteins by oligonucleotide-directed mutagenesis [6]. For example, the specificities of the proteolytic enzyme subtilisin [7] and the pyridoxal phosphate-dependent enzyme aspartate aminotransferase [8] have been successfully altered. More recently, a method has also been developed which allows the genetic insertion of unnatural amino acids into proteins at specific locations [9]. This approach may increase our ability to rationally manipulate protein function through the substitution of amino acids with novel electronic and structural properties. Random mutagenesis of proteins in combination with genetic selections and screens also holds promise for generating proteins with novel functions. Genetic selections have been used to modify the specificity of DNA binding proteins [10] and to generate heat stable proteins [11–13]. Successes are also being realized in the de novo design of peptides and proteins of predefined structure and/or functions [14–17]. An exciting recent approach to the design and generation of novel catalysts involves exploitation of the vast binding repertoire of the immune system to generate highly selective catalysts. Antibodies have been generated that selectively catalyze hydrolytic [18–20], photochemical [21], elimination [22] and pericyclic reactions [23,–24].

2 Semisynthetic Proteins

Another powerful approach to the design of catalysts is the generation of semisynthetic proteins. Semisynthesis combines elements of both the biological and chemical strategies described above. The chemically modified protein, which is a hybrid of a catalytic and a binding domain, has new properties which

reflect both constituents. This approach exploits the innate binding specificities or catalytic activities of proteins, and the diverse chemical functionality available from synthetic chemistry. Keys to the design and generation of semisynthetic proteins are: (1) the ability to selectively modify a complex multifunctional protein at the desired site with the chemical moiety of interest and (2) fusion of the synthetic and protein constituents such that the desired properties of each are reflected in the hybrid enzyme.

2.1 Thiol-subtilisin

One of the earliest approaches toward the design of semisynthetic proteins involved the chemical mutation of enzymatic residues. A classic example is the modification of the active site serine of the protease subtilisin to cysteine (S221C) [25–28]. The serine was converted to the thiolester by treatment with methanesulfonyl chloride and subsequent displacement with thiolacetic acid. The thiolester was then hydrolyzed to afford cysteine 221. Although the resulting “thiol-subtilisin” lacked protease activity, it could still bind and hydrolyze activated peptide esters. Similar low levels of catalytic activity have been observed with semisynthetic “thiol-trypsin” [29] and “hydroxy-papain” [30].

Kaiser and coworkers have exploited the ability of thiolsubtilisin to catalyze transesterification reactions as a method for selective coupling of peptide fragments. The active site cysteine can be acylated by a C-terminal peptide ester. The resulting thiolester can then be displaced by the N-terminal amino group of a second peptide fragment to form ligated product [31]. This condensation can also be carried out with wild type subtilisin or other proteases, but only in the presence of organic solvents or other conditions which suppress amidase activity [32].

2.2 Flavo-papain

Kaiser and coworkers expanded the scope of semisynthesis by chemically introducing cofactors into the active sites of well characterized hydrolytic proteins to generate enzymes with new functions. In this way, some of the binding properties of the protein were retained, and proximity of substrate to the covalently attached coenzyme resulted in catalytic activity. For example, the active site cysteine 25 of papain was selectively alkylated by a bromoacetyl flavin derivative [33]. The hydrophobic binding pocket of papain is large enough to accommodate the cofactor as well as potential substrates, and the “flavopapain” proved to be an efficient oxidoreductase for hydrophobic *N*-alkyl-1,4-dihyronicotinamide substrates. Although this work demonstrated that it is possible to rationally obtain new chemical activities from existing enzymes, the inherent catalytic activity of the enzyme as well as its ability to selectively bind peptides were destroyed.

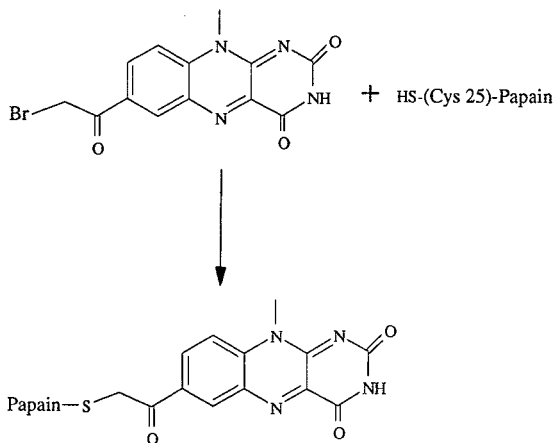


Fig. 1. Derivatization of papain to generate flavo-papain

2.3 Semi-Synthetic Antibodies

The semisynthetic approach is limited by the availability of enzymes which can be selectively modified at a unique residue. Indiscriminate modification of an enzyme might damage the active site, or lead to nonselective chemical reactions away from the binding site. One solution to this problem involves the use of cleavable affinity labels to selectively introduce a unique chemical handle into a protein which can be subsequently derivatized [34, 35]. The affinity label consists of a reactive electrophile linked to a substrate analog by a cleavable tether. The substrate analog delivers the reactive group into proximity of the active site, and the electrophile then alkylates a specific lysine, tyrosine, serine, or histidine residue nearby. Subsequent reduction of a disulfide or hydrolysis of a thiolester in the linker then produces a thiol-labelled protein. The thiol "handle" can act as a nucleophilic catalyst itself or can be further modified with other functional groups, such as cofactors, reporter molecules, or therapeutic agents. This is a general strategy which allows one to selectively derivatize any protein (or other biopolymer) near its binding site, thereby extending the semisynthetic approach to proteins which lack residues which can be selectively modified. Moreover, the use of cleavable affinity labels requires very little structural information about the protein.

This strategy has been used to affinity label an antibody, MOPC 315, in order to introduce catalytic residues into the combining site. The introduction of a nucleophilic thiol near the active site accelerates ester thiolysis 60,000 fold over background [34]. Other experiments have shown that an imidazole attached to the antibody via the free thiol can catalyze ester hydrolysis [35]. A fluorophore was also introduced at the periphery of the antibody combining site to generate a semisynthetic biosensor [34]. It should be possible to extend this technique so

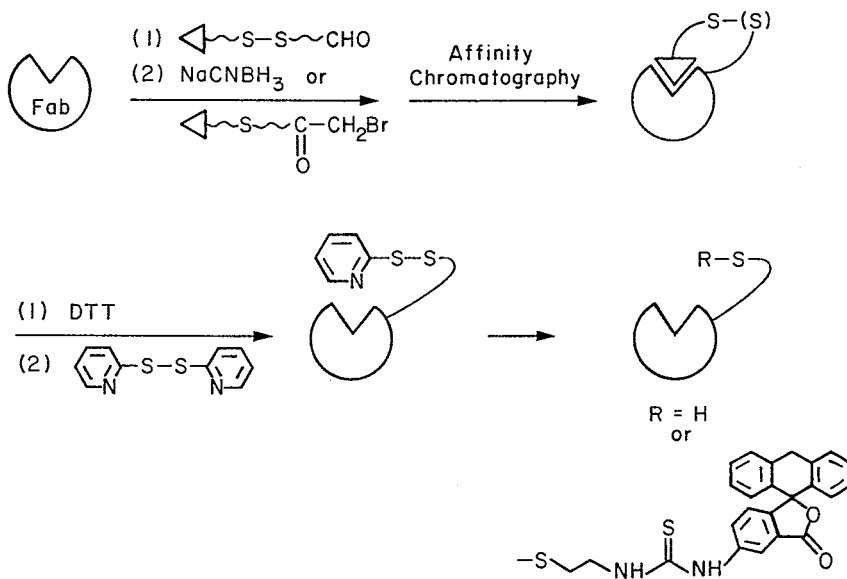


Fig. 2. Introduction of a thiol into an antibody combining site via a cleavable affinity linker, and its subsequent derivatization with imidazole and fluorescein derivatives

that, in theory, catalytic activity could be introduced into any antibody that selectively binds a substrate of interest.

The exquisite binding specificity of antibodies has also made them ideal vehicles for delivering toxins [36], enzymes [37, 38], and radioisotopes [39] to a variety of physiological targets. Antibodies have been derivatized nonselectively with crosslinking reagents specific for the ϵ -amino groups of lysine [40] or via oxidation and subsequent reductive amination of sugar residues. Bi-specific antibodies have also been constructed by a disulfide exchange reaction between two different Fab fragments [41].

An antifibrin monoclonal antibody has been conjugated to two thrombolytic enzymes, urokinase and tissue plasminogen activator (Tpa) [37, 38] by chemical crosslinking and gene fusion. The resulting chimeric adducts were 100 times as active as urokinase and 10 times as active as Tpa in assays for the lysis of fibrin monomers.

3 Sequence Specific Hybrid Nucleases

Previous examples of semisynthetic proteins focused on the chemical introduction of catalytic groups into existing proteins to generate new catalysts with the binding specificity of the native protein. Alternatively, one can generate selective catalysts by chemically introducing new binding domains into the catalytic sites of relatively nonspecific enzymes. The synthetic component in this case serves to selectively deliver the catalytic domain of the enzyme to the substrate of interest.

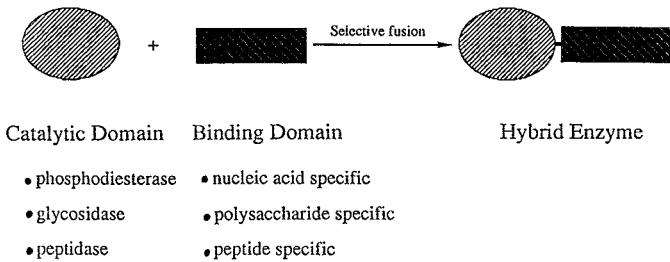


Fig. 3. The combination of binding and catalytic domains to generate hybrid enzymes

3.1 Hybrid Nucleases as Tools for the Sequence-Selective Cleavage of RNA and DNA

The synthesis of catalysts with the ability to sequence-specifically cleave RNA or DNA would provide useful tools for molecular biology and genomic mapping. In spite of the obvious value of naturally occurring type II restriction enzymes for gene isolation, DNA sequencing and recombinant DNA technology, their utility for analyzing very large DNAs is limited by their relative lack of specificity. Most restriction enzymes have four to six base recognition sites, and have cleavage frequencies of once in every 136 to 2080 basepairs, respectively. Since a single mammalian chromosome can contain over 100 million basepairs, these enzymes generate far too many fragments to be analyzed by current gel electrophoretic methods. Even those few restriction enzymes with the rarest recognition sequences [42], which cleave DNA once every million bases and are used to map relatively small genomes, cleave very large chromosomes too frequently to be readily used for the ordering of mammalian DNA. In addition, restriction enzymes do not in general cleave single-stranded DNA, although adaptor deoxyribonucleotides have been used to modify the specificity of the FokI restriction enzyme for specific cleavage of single-stranded DNA [43]. Hybrid nucleases with recognition sites of 15 nucleotides (nt) would statistically cleave single-stranded DNA once per 540 million base pairs and duplex DNA once per 270 million base pairs and should therefore be of considerable value for analyzing the structure of large DNAs.

Enzymes have not yet been isolated that are capable of cleaving RNA with specificities like those of restriction enzymes. Currently, two methods exist for specific cleavage of RNA: cleavage of RNA/DNA hybrids by RNase H [44,45] and cleavage of RNA by ribozymes [46]. The former method is limited by the fact that RNase H will cleave hybridized hexamers with as few as three complementary base pairs [44]. A sequence specific RNase with a defined recognition site of 5–20 base pairs would allow for the cleavage of small and large RNAs at unique sites. Such sequence-specific RNases would aid in manipulations of RNA for cloning, in isolating RNA fragments for sequencing, and in manipulating RNAs in studies of RNA secondary and tertiary structure

as well as RNA processing and splicing (for example, in analyzing psoralen crosslink sites) [47, 48].

Current approaches to the generation of sequence specific nucleases have focused on tethering a synthetic DNA-cleaving moiety to a synthetic or natural nucleic acid binding domain. Oxidative cleaving agents like EDTA-Fe(II) or Cu-phenanthroline have been coupled to DNA binding antibiotics [49], oligonucleotides [50–52] and peptides [53, 54] in order to generate reagents that sequence-selectively cleave DNA and RNA.

We have undertaken the development of semisynthetic enzymes capable of sequence specifically hydrolyzing large naturally occurring DNA and RNA molecules. The nonspecific phosphodiesterases, staphylococcal nuclease and RNase A, have been selectively derivatized with an oligonucleotide of defined sequence near the enzyme active site. The high affinity and specificity inherent in Watson–Crick base-pairing interactions directs the hydrolytic activity of the hybrid nuclease to predefined target sites on single-stranded DNAs, RNAs, and duplex DNAs. This approach offers several advantages over oxidative cleavage, most notably the high efficiency of the enzymatic reaction and the fact that hydrolysis generates termini that can be used in subsequent enzymatic reaction.

3.2 Design Considerations

The design of the hybrid nucleases was guided by several considerations:

1. The oligonucleotide binding domain must be crosslinked in the correct orientation relative to the enzyme such that the hybridized substrate is productively aligned with respect to the enzyme's active site. The crosslinking reaction must therefore be highly selective for unique locations on both biomolecules. Moreover, it should be possible to carry out the crosslinking reaction in high yield and under mild conditions so that both biomolecules retain activity.
2. Detailed structural and mechanistic data are required to determine at what sites the two domains should be crosslinked.
3. The enzyme should not hydrolyze or impair binding of the oligonucleotide binding domain to the target sequence. Conversely, the binding domain should not significantly inhibit enzymatic activity.
4. Both the catalytic and binding domains should be stable and readily available. Ideally, the gene for the protein should be cloned and efficiently expressed so that the protein can be readily modified via genetic methods.
5. The synthetic route to the hybrid nucleases should allow the rapid generation of hybrid nucleases with a variety of specificities.
6. In order for the hybrid enzyme to cleave at the desired sequence with high selectivity, the k_{cat}/K_m of the hybrid enzyme for the target sequence must be considerably higher than that of native enzyme for nonspecific sites.

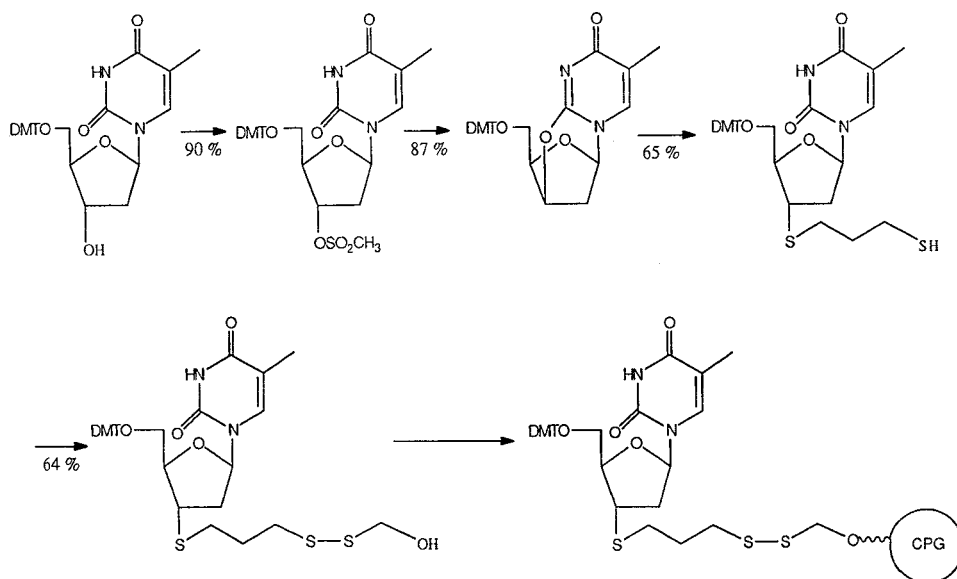
4 Oligonucleotide-Directed Staphylococcal Nuclease

The synthesis of the hybrid nucleases required a highly selective chemical method for crosslinking the oligonucleotide to the enzyme under mild conditions and in high yield. This was accomplished via the selective introduction of thiols into unique positions in both the oligonucleotide and the enzyme, and their subsequent crosslinking by disulfide exchange.

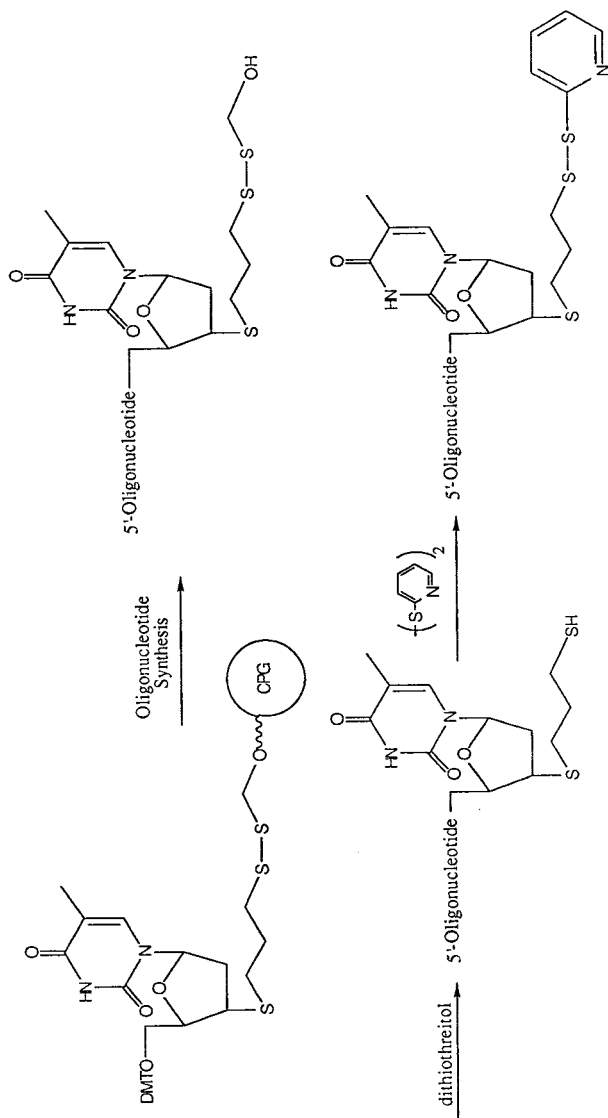
4.1 Oligonucleotide Binding Site

The base-pairing interactions between complementary DNA or RNA oligomers is characterized by high affinity and specificity. Under appropriate experimental conditions [55], discrimination between nucleic acid sequences that differ by as little as a single nucleotide is possible (ΔT_m [melting temperature] of $\sim 10^\circ\text{C}$ has been observed for 14-mers). This specific binding interaction has been exploited in the design of labeled hybridization probes for the detection and cloning of specific genes [56], in specific priming of DNA for sequencing, polymerase chain reaction [PCR] and mutagenesis [57, 58], in the regulation of RNA transcription and degradation of RNA by antisense oligonucleotides [59], and in the selective cleavage of nucleic acids [50–52]. These experiments suggest that an oligodeoxyribonucleotide of defined sequence should serve as a highly specific synthetic binding site for delivering the hydrolytic activity of staphylococcal nuclease or RNase A to DNA or RNA substrates.

Solid phase DNA synthetic methods make possible the rapid synthesis of oligonucleotides of any defined sequence (< 100 bp). Existing methods for the



Scheme 1. Synthesis of 3'-thiolated thymidine and its attachment to controlled pore glass (CPG)



Scheme 2. Synthesis of S-thiopyridyl activated oligonucleotides

automated solid-phase phosphoramidite or phosphotriester synthesis of oligonucleotides can be modified to permit the facile generation of oligonucleotides containing free 3'-thiols [60]. The 3' hydroxyl of 5'-dimethoxytrityl thymidine was substituted with a thiol, and subsequently protected as a disulfide by treatment with 2'-S-thiopyridyl mercaptoethanol. This process regenerates a free hydroxyl which can be coupled to controlled pore glass (CPG) by standard methods. This approach has the advantage that the 3'-thiol is introduced prior to DNA synthesis. In addition, the thiol can either be introduced directly at the 3' position of the deoxyribose ring, or it can be attached to the 3' hydroxyl through linkers of varying lengths and compositions (we have introduced tethers ranging in lengths from 2.5 Å to 20 Å) [61]. Yields of oligonucleotides synthesized using this support are similar to those from commercially prepared support lacking the disulfide.

After standard synthesis and deprotection procedures the disulfide bond is cleaved with dithiothreitol. The reduced oligonucleotide is purified and subsequently reacted with 2,2'-dithiodipyridine to afford the 3'-S-thiopyridyl oligonucleotide. This modified oligonucleotide can be stored indefinitely at -20°C and can be readily crosslinked to any compound containing an accessible free thiol.

4.2 Properties of Staphylococcal Nuclease

Staphylococcal nuclease is an ideal candidate for semisynthetic studies. It is a well characterized and stable enzyme consisting of a single polypeptide chain 149 amino acids in length [62–65]. There are no intrachain disulfides or free cysteines. The enzyme hydrolyzes the phosphodiester bonds of either RNA or DNA to generate 3'-phosphate and 5'-hydroxyl termini. The rate of hydrolysis of single-stranded DNA by staphylococcal nuclease is estimated to be approximately 10^{16} higher than the aqueous background rate [66]. The hydrolysis of single-stranded substrate is much faster than that of double stranded, and cleavage is relatively nonspecific with a preference for thymidine rich regions ($\text{T} > \text{A} \gg \text{C}, \text{G}$) [80]. Ca^{2+} is required for activity, providing a mechanism for rapidly modulating enzyme activity [67]. This efficient on/off switch allows the synthesis of hybrid nuclease without hydrolysis of the crosslinked oligonucleotide binding domain. The oligonucleotide-nuclease adduct can be activated by addition of Ca^{2+} after hybridization to the target sequence.

The structure and mechanism of staphylococcal nuclease have been elucidated from a series of chemical, physical and genetic studies [62–68]. An X-ray crystal structure of a staphylococcal nuclease-diphosphothymidine (pTp)- Ca^{2+} complex has been determined to 1.5 Å [68]. The pyrimidine ring of the inhibitor pTp fits into a hydrophobic pocket at the enzyme surface and the 5'-phosphate is near Arg35 and Arg87. Glu43 is thought to act as a general base for activation of the attacking water molecule whereas Arg35, Arg87, and Ca^{2+} stabilize the trigonal bipyramidal transition state configuration [66, 68]. The gene for the nuclease has been cloned and the protein has been efficiently expressed in *E. coli*

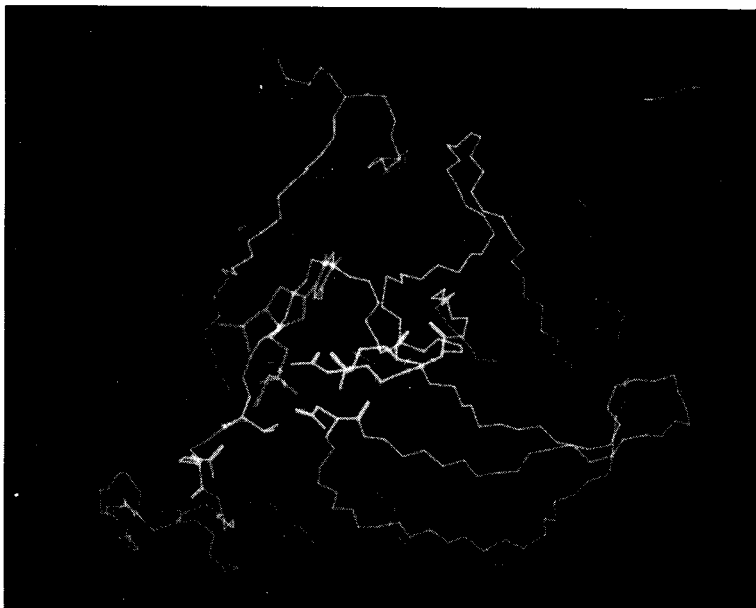


Fig. 4. X-ray crystal structure of staphylococcal nuclease-pTp-Ca²⁺ complex (1.5 Å) [Ref. 68].

behind an ompA leader sequence [69, 70] and is secreted into the periplasm. Examination of the geometry of the bound inhibitor pTp suggested that coupling the 3' thiol of the oligonucleotide binding domain to a cysteine at position 116 on the enzyme surface should align a hybridized DNA or RNA substrate with the enzyme active site.

4.3 Use of Cysteine in Protein Engineering

Cysteine is unique among the 20 commonly occurring amino acids by virtue of the nucleophilicity of the thiol group and its ability to form disulfide bonds. Consequently, thiols have been site-selectively incorporated into proteins in order to (a) provide a handle for selectively derivatizing proteins with additional functional groups [34, 35]; (b) generate new disulfide bonds which act to stabilize or probe protein structure [71–73]; (c) function as nucleophilic catalytic groups [25, 26]; and (d) introduce regulatory switches [74, 75] of enzymatic activity. The latter experiment is applicable to the development of hybrid nucleases since it offers a means to repress catalytic activity until after substrate is bound by the hybrid enzyme. For example, a cysteine has been introduced into the binding cleft of staphylococcal nuclease that can block substrate binding by forming a disulfide bond with thiol-reactive reagents or by chelating Cu²⁺ or Hg²⁺⁷⁵. Activity can be rapidly restored by the addition of reducing agents or metal chelators. In a second example, redox active disulfide bonds have been engineered into T4 lysozyme to block access of substrate to the active site [74].

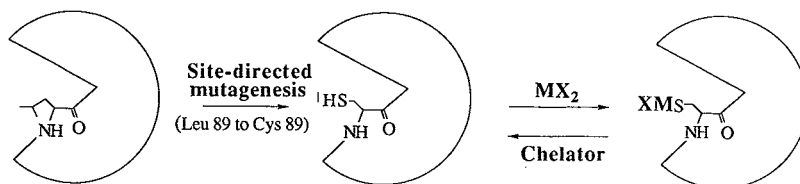


Fig. 5. The introduction of cysteine into the binding pocket of staphylococcal nuclease and its reversible derivatization

4.4 Synthesis of the Hybrid Nuclease

A free thiol was introduced into staphylococcal nuclease by replacing the lysine at position 116 with a cysteine via oligonucleotide-directed mutagenesis [76, 77]. The mutant protein, K116C, was isolated as a dimer. Monomeric enzyme could be obtained by reduction of the dimer with dithiothreitol. The k_{cat} and K_m of the mutant enzyme ($K_m = 8.1 \mu\text{g DNA/mL}$, $V_{\text{max}} = 0.31 \Delta\text{abs}_{260}(\mu\text{g nuclease})^{-1} \text{min}^{-1}$) were determined to be similar to those of wild-type enzyme ($K_m = 2.76 \mu\text{g DNA/mL}$, $V_{\text{max}} = 0.66 \Delta\text{abs}_{260}/(\mu\text{g nuclease})^{-1} \text{min}^{-1}$) [77].

The monomeric enzyme was crosslinked to the 3'-S-thiopyridyl oligonucleotide to produce the hybrid nuclease in high yield (Fig. 6). The crosslinking reaction (< 10 minutes) can be monitored by following the release of thiopyridyl anion at 343 nm. This reaction had been used previously to nonselectively crosslink RNase A to staphylococcal nuclease and resulted in a 30% recovery of active crosslinked enzymes [78]. The disulfide exchange can be carried out in buffered solution, pH 4–pH 9, and is considerably more selective than methods which rely on amine or carboxyl specific modifying reagents [79]. A flexible tether was incorporated to allow some movement between the hybridized substrate and the nuclease active site. The oligonucleotide-nuclease adduct can be easily purified via either cation or anion exchange chromatography since the combination of cationic nuclease and anionic oligonucleotide gives it a significantly altered retention time relative to either of the starting materials [77]. Treatment of the purified product with dithiothreitol regenerates the nuclease and the oligonucleotide. The adduct is stable indefinitely at -20°C in the presence of the Ca^{2+} chelator EGTA.

4.5 Sequence-Selective Cleavage of Unstructured Substrates

The hybrid nuclease was assayed for its ability to selectively cleave a 5'- ^{32}P end-labelled 64 nucleotide (nt) synthetic DNA oligomer containing a complementary binding sequence [79]. The assay involved annealing the hybrid nuclease to the substrate for 5 minutes at 60°C in the absence of Ca^{2+} . The reaction was then cooled to the desired temperature and CaCl_2 was added to initiate cleavage. EGTA was then added to quench the reaction, and the products were analyzed by polyacrylamide electrophoresis and subsequent autoradiography.

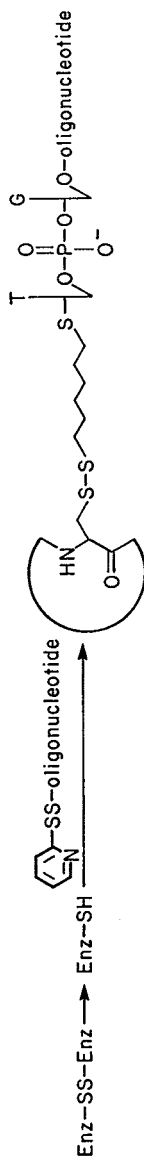


Fig. 6. Synthesis of the hybrid nuclease. Dimeric K116C staphylococcal nuclease is reduced to afford the monomer, which is subsequently crosslinked with the 3'-S-thiopyridyl oligonucleotide to generate the hybrid nuclease.

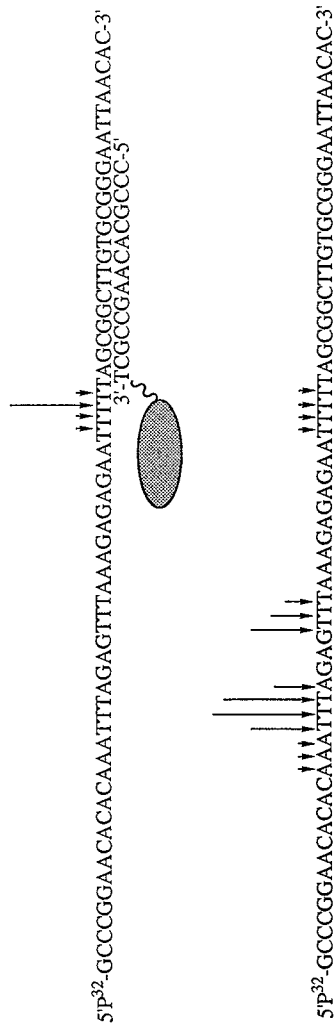


Fig. 7. Histogram of DNA cleavage by hybrid nuclease (**top**) and free nuclease K116C (**bottom**). The heights of the arrows represent the relative cleavage intensities at the indicated bases

Initial experiments demonstrated that the hybrid nuclease could cleave the 64 nt DNA substrate with high selectivity adjacent to the oligonucleotide binding site [77] (Fig. 7). In contrast, the underivatized Cys 116 mutant enzyme, either in the absence or presence of the 15 nt oligomer, cleaves relatively nonspecifically at T-rich sites. Unfortunately, selective cleavage by the hybrid enzyme can only be achieved within a narrow range of temperatures (0–5 °C), reaction times (< 5 s) and substrate concentrations (< 15 nM). When conditions outside these ranges are used the inherent nonselective hydrolytic activity of the enzyme dominates oligonucleotide-directed cleavage. In addition, the hybrid nuclease functioned only as a stoichiometric reagent.

4.6 Optimization of the Hybrid Nuclease

The incorporation of an oligonucleotide binding domain into staphylococcal nuclease allows delivery of the enzyme to specific target sequences. However, the enzyme can still cleave at other T-rich sites by virtue of its native binding affinity. Consequently, cleavage by the hybrid nuclease reflects the specificities of *both* the oligonucleotide and staphylococcal nuclease. In order to increase the specificity of the hybrid enzyme as well as realize catalytic turnover, the enzyme's intrinsic binding affinity to DNA/RNA must be reduced relative to the binding affinity of the attached oligonucleotide. To this end mutations have been introduced into the active site of staphylococcal nuclease in order to decrease the enzyme's k_{cat}/K_m [77] and reduce the rate of nonspecific (oligonucleotide independent) hydrolysis. Cleavage at sites adjacent to the oligonucleotide binding site should be relatively unaffected, since hybridization makes the effective concentration of nuclease very high.

A mutation, Y113A, was introduced into the binding site of the K116C staphylococcal nuclease [80]. The K_m of the Y113A, K116C mutant was increased by a factor of 4.2, the V_{max} was decreased by a factor of 190 and the V_{max}/K_m was reduced by a factor of 790. Examination of the 1.5 Å crystal structure suggests that the decrease in k_{cat}/K_m may be due to the removal of a π -stacking interaction between Tyr 113 and the bound purine or pyrimidine base. The mutation did not significantly alter the preference of the enzyme for hydrolysis at T-rich sites.

Hybrid enzymes constructed from either the K116C or the Y113A, K116C nuclease were assayed for their ability to selectively hydrolyze a 78 nt substrate. The K116C hybrid enzyme showed little or no specificity when assayed at temperatures greater than 20 °C, at reaction times longer than 10 s or at substrate concentrations greater than 20 nM. In contrast the Y113A, K116C mutant hybrid enzyme sequence selectively hydrolyzed DNA at temperatures as high as 75 °C and over reaction times as long as one hour [80].

4.7 Cleavage of Structured DNA Substrates

The selective cleavage of complex naturally occurring single-stranded DNA's requires hybrid nucleases that can hydrolyze DNA containing considerable

secondary and tertiary structure with high specificity. Templates with substantial secondary structure near or at the target site cannot be selectively hydrolyzed by hybrid enzymes lacking the binding pocket mutation because the elevated temperatures which are required to eliminate structure and make the site accessible to the nuclease also greatly increase nonselective hydrolysis. However, hybrid nucleases that contain the binding pocket mutation (Y113A) and carry out efficient selective cleavage at elevated temperatures should be able to hydrolyze structured DNA. In order to test this notion a 63 nt substrate was designed which has the target thymidines constrained within the base-paired region of a hairpin loop [80]. At 0 °C the Y113A, K116C hybrid nuclease did not cleave at the desired site, although nonspecific hydrolysis did occur towards the 5' terminus of the 63 nt substrate. However, at 65 °C, which is above the melting temperature for the hairpin, cleavage does occur adjacent to the oligonucleotide binding site.

The ability of the Y113A hybrid nucleases to cleave short structured DNA substrates suggested that the hybrid nuclease might selectively cleave much

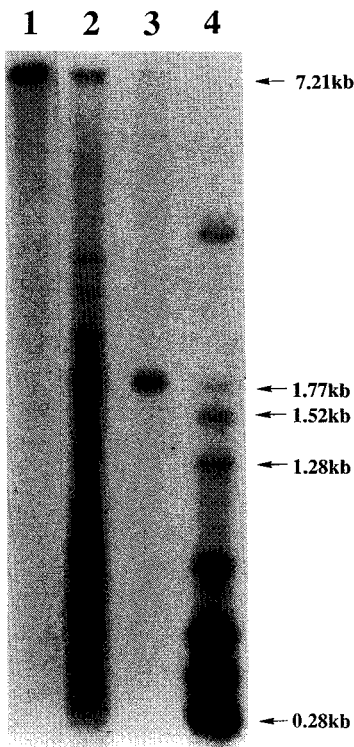


Fig. 8. Cleavage of M13mp7 by a hybrid nuclease. *Lane 1*, M13mp7; *lane 2*, M13mp7 treated with Y113A, K116C staphylococcal nuclease; *lane 3*, selective hydrolysis of M13mp7 by a hybrid nuclease; *lane 4*, molecular weight standards

longer naturally occurring single-stranded DNAs. We chose to test this hypothesis using single-stranded M13mp7 [81], a 7238 circular DNA, as a substrate [80]. This DNA was designed with a short hairpin region containing a number of restriction sites, which allow it to be linearized and end labelled with ^{32}P -ATP so that cleavage specificity can be analyzed to nucleotide resolution by polyacrylamide gel electrophoresis. When linearized M13mp7 was digested with an equimolar concentration of a 22 nt hybrid enzyme lacking the Y113A mutation, nonspecific cleavage predominated. In contrast, the same 22 nt hybrid nuclease containing the Y113A mutation selectively hydrolysed the substrate at the desired sequence. Moreover at 0 °C, cleavage occurred primarily at just one phosphodiester bond. Importantly, these results demonstrate that the hybrid nuclease can bind and cleave one phosphodiester bond within a large structured DNA without hydrolyzing the many other susceptible T-rich sites which are present.

4.8 Catalytic Turnover by Hybrid Nucleases

The ability of the Y113A mutant hybrid nuclease to efficiently carry out selective cleavage at elevated temperatures and over long reaction times suggested that cleavage could occur at the T_m of the oligonucleotide domain. Since under these conditions, the bound and free hybrid nucleases are in rapid exchange, the nuclease should be able to catalytically cleave the target DNA. Hybrid nucleases containing the Y113A, K116C double mutant, or a L37A, Y113A, K116C triple mutant (which has very similar properties to those of the double mutant) were assayed for their ability to catalytically hydrolyze substrate DNA [80].

A hybrid nuclease constructed from the triple mutant and containing a 15 nt binding domain [T_m (calc.) = 62 °C] was able to catalytically cleave a 78 nt substrate adjacent to the oligonucleotide binding domain at 65 °C. A Lineweaver-Burke analysis of the initial rates afforded a k_{cat} of 1.5 min $^{-1}$ and a K_m of 120 nM.

The catalytic properties of the hybrid nucleases can be rapidly altered by varying the length and sequence of the oligonucleotide binding domain. A hybrid nuclease with an 8 nt oligonucleotide was catalytic over a lower range of temperatures (30–60 °C) while a 19 nt hybrid nuclease showed catalytic activity at temperatures between 55 °C and 72 °C (Fig. 9). The 8 nt hybrid nuclease showed more nonspecific cleavage, but also a 20 fold higher rate of turnover. The effects of mismatches in the oligonucleotide binding site on the catalytic properties of the hybrid nucleases reflect previously reported effects of mismatches on DNA hybridization [55]. These results are again consistent with the notion that dissociation of hybrid enzyme is the rate limiting step.

The ability of the hybrid nucleases to catalytically cleave DNA in a selective fashion may appear somewhat surprising given that the crosslinked oligonucleotide can be digested by Ca^{2+} -activated staphylococcal nuclease. Consequently, the stability of the oligonucleotide-nuclease adduct towards autolysis

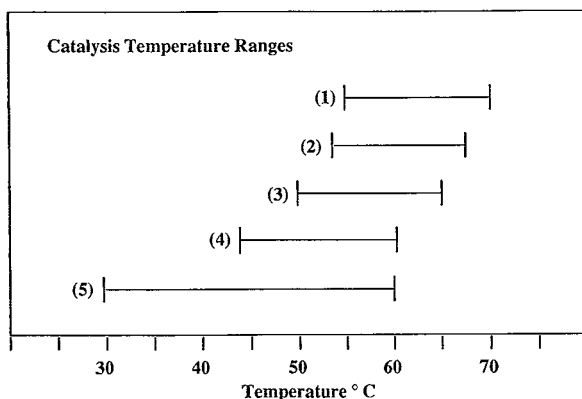


Fig. 9. The temperature ranges for catalytic turnover for Y113A, K116 C hybrid nucleases with differing binding domains. (1) 19 nt binding domain; (2) 15 nt binding domain; (3) 15 nt binding domain with a 3' terminal mismatch; (4) 15 nt binding domain with an internal mismatch; (5) 8 nt binding domain

at elevated temperatures and over extended reaction times was investigated by 5' end-labelling the 19 nt binding domain of a hybrid nuclease (Y113A, K116C mutant) with ^{32}P . The labelled adduct was incubated with Ca^{2+} at 37°C in the absence of substrate and the course of the incubation was monitored by electrophoresis. No degradation was observed after 30 minutes. Resistance to intramolecular autolysis may be due to an inability of the oligonucleotide to align itself in the correct 5' to 3' orientation for cleavage in the enzyme active site.

The ability of hybrid enzymes to catalytically cleave DNA demonstrates the extent to which the nuclease's hydrolytic activity can be selectively delivered to specific sequences within a DNA substrate. Cleavage by the hybrid enzyme is selective even though a significant fraction of the nuclease is free in solution under conditions which would lead to complete and indiscriminate degradation of DNA by wild type enzyme. A combination of chemical mutagenesis (introduction of an oligonucleotide) followed by biological mutagenesis (binding site mutations) can therefore be used to rationally modify the specificity of an enzyme while still maintaining its desired catalytic properties.

4.9 Alteration of the Interdomain Linkage

In a number of cases, especially with unstructured substrate DNAs, the hybrid nuclease cleaves a number of phosphodiester bonds adjacent to the target site as well as sites distant from the hybridization site. This result was initially attributed to the flexibility of the tether linking the enzyme and oligonucleotide. Hybrid nucleases containing short linkages (-S-S-) should be less flexible than those with longer tethers, and this might result in less non-selective cleavage

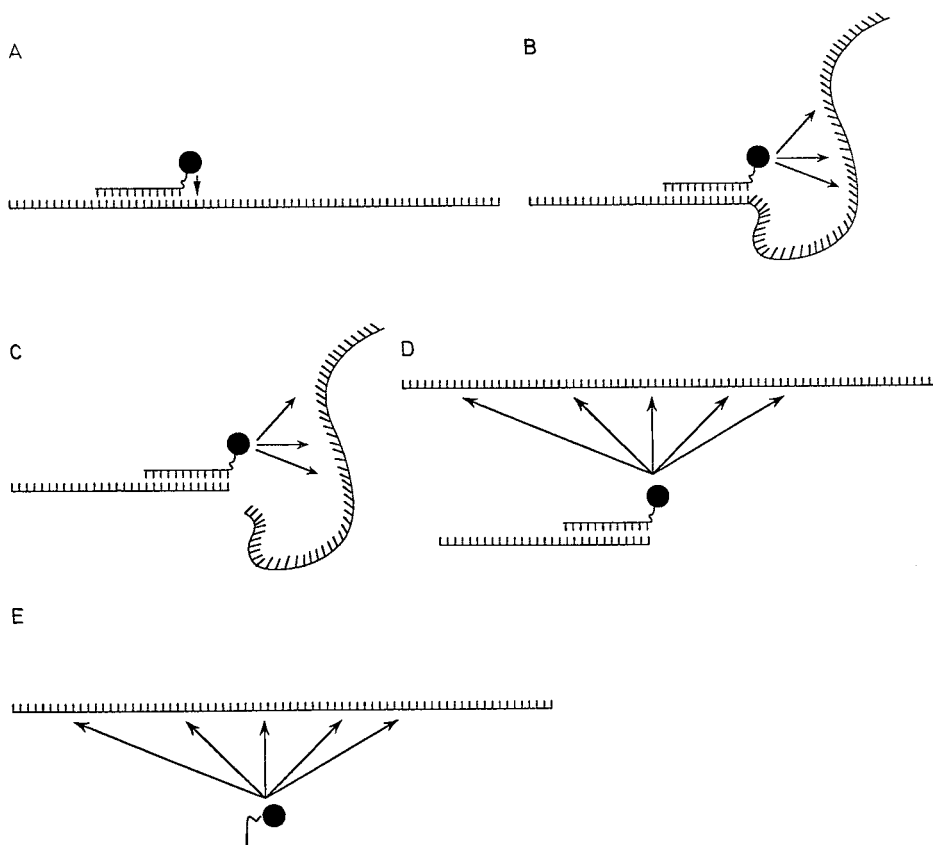
away from the target site and limit cleavage to one phosphodiester bond. To this end the nucleoside analogue 3'-mercapto-5'-(4,4'-dimethoxytrityl) thymidine was synthesized and attached to controlled pore glass [80]. Oligonucleotides synthesized using this support have been coupled to both the K116C and the Y113A, K116C mutants to generate hybrid enzymes with only a disulfide linkage between the catalytic and binding domains.

These hybrid enzymes were compared to those linked by 1,6-hexanedithiol tethers by assaying their cleavage with several different substrates. Only slight differences were observed in cleavage specificity [80]. These results suggest that the structure and sequence of the substrate is a primary factor in determining which phosphodiester bonds are hydrolyzed, and that the hybrid enzyme-substrate complex has considerable flexibility which is independent of the length of interdomain linkage.

4.10 Mechanisms for Specific vs Nonspecific Hydrolysis

Although the double (Y113A, K116C) and triple mutant (L37A, Y113A, K116C) hybrid enzymes cleave DNA both catalytically and under a wide range of conditions, this cleavage is accompanied by some nonspecific hydrolysis. This undesired reactivity can range between 10% to 60% of the specific cleavage, depending on the reaction conditions, the substrate, and the composition of the hybrid nuclease. Understanding the nature of nonspecific cleavage should aid in engineering hybrid nucleases that approach restriction enzymes in their specificity and cleavage efficiency. Nonspecific cleavage associated with the nuclease can occur via a number of mechanisms (Scheme 3). The nuclease can bind and cleave DNA in an intermolecular reaction which is independent of the oligonucleotide domain and which can occur either before (Scheme 3, E) or after hybridization and cleavage of the target sequence (Scheme 3, D). Nonspecific cleavage can also occur in an intramolecular fashion, in which hybridization first localizes the nuclease at the target site. Conformational flexibility or secondary structure in the substrate at the target site may then make upstream 5' T-rich sites accessible to cleavage by the nuclease (Scheme 3, B). Alternatively cleavage at the target site can be followed by secondary cleavage at other T-rich sites by a processive mechanism in which the enzyme does not dissociate from substrate a significant component of single-stranded DNA cleavage by free staphylococcal nuclease does occur via a processive mechanism) (Scheme 3, C).

In order to gain insights into the mechanism of nonspecific cleavage, reactions were carried out in the presence of an excess of poly (dA) [80]. Over reaction times of less than 15 seconds, and at temperatures which do not allow turnover, the addition of a 200-fold excess (w/w) of unlabelled poly (dA) DNA as a scavenger did not effect the amount of nonspecific cleavage produced by hybrid enzymes containing the Y113A mutation. However, similar treatment reduces the amount of nonspecific hydrolysis by hybrid enzymes which lack the binding pocket mutation or by a noncognate hybrid enzyme which lacks a complementary binding domain. These results suggest that intermolecular



Scheme 3. Mechanisms for specific and nonspecific cleavage

cleavage contributes significantly to the nonspecific cleavage associated with hybrid enzymes lacking the binding pocket mutations, but that nonspecific cleavage by the Y113A-containing enzymes is primarily due to intramolecular hydrolysis. This supports the conclusion that the improved specificities of the Y113A hybrid enzymes arise from the mutants' lowered affinity for DNA substrate which allows discrimination between nonspecific intermolecular hydrolysis and hydrolysis which is directed by the oligonucleotide domain. Nonspecific cleavage, however, is considerably reduced when structured DNA [40] or RNA substrates [82] are used, and therefore also appears to be associated with the secondary structure of substrate.

4.11 Selective Hydrolysis of Duplex DNA by a Hybrid Nuclease

The sequence-selective hydrolysis of double-stranded DNA presents challenges which are distinct from those which are encountered with single-stranded

DNAs. The hybrid enzyme must (1) hybridize to a complementary sequence within the double helix and (2) cleave both strands of the double helix. Hybridization of the oligonucleotide to the target sequence by Watson–Crick base pairing interactions requires disruption of the double helix. One way to avoid the need to locally denature the DNA is by taking advantage of Hoogsteen base pairing of the oligonucleotide to the target sequence to form triple helical DNA. This approach has been used successfully to deliver oxidative cleavage functionalities to specific sites within DNA substrates [51, 52]. However, triple helix formation is currently limited to polypurine-polypyrimidine target sequences.

Another strategy to introduce an oligonucleotide at a complementary site in a duplex DNA involves D-loop formation. In this process supercoiled DNA spontaneously and stably incorporates complementary single strands of DNA [83]. This uptake is accelerated if the supercoiled DNA is partially denatured with base [84, 85] (the reaction can also be catalyzed by the recombination enzyme *rec A* [86, 87]). D-loop formation has been used to deliver oligonucleotides to complementary sequences within double-stranded DNA for sequencing of duplex DNA [85] and as hybridization probes [84].

The ability of the hybrid nuclease to sequence-specifically hydrolyze supercoiled DNA via D-loop formation was assayed with pUC 19, a 2686 base-pair plasmid [89]. The sequence of the oligonucleotide binding domain of the hybrid nuclease was chosen so that it would deliver the attached nuclease to a site within the plasmid that contained thymidines on both strands adjacent to the bound hybrid nuclease [88]. Hybrid nucleases were synthesized both with and without the Y113A mutation, and with cysteine at either positions 84 and 116. Plasmid pUC 19 was partially denatured with 3.5 mM NaOH for 10 min. The DNA solution was then neutralized and hybrid nuclease was added.

In order to determine the extent of specific cleavage, the reaction products were treated with the restriction enzyme, *Hind III*, in order to generate discrete fragments which could be compared to known standards. Inspection of the ethidium bromide-stained agarose gel revealed that all hybrid nucleases cleaved DNA at the predicted target site, but that the hybrids lacking the Y113A mutation hydrolyzed substrate in much higher yield than either the K113C, Y116A or the K84C, Y113A enzyme. Presumably the greater catalytic activity of the single mutant enzyme allows it to more readily cleave both strands of the duplex prior to dissociation. Selective cleavage at other sites on pUC 19 was less efficient and showed higher levels of nonselective hydrolysis. The susceptibility of the original site may be due to its T-rich nature or to the presence of a region of secondary structure which facilitates denaturation. Considerable work re-



Fig. 10a. D-loop formation and subsequent hydrolysis of duplex DNA

1 2 3 4 5

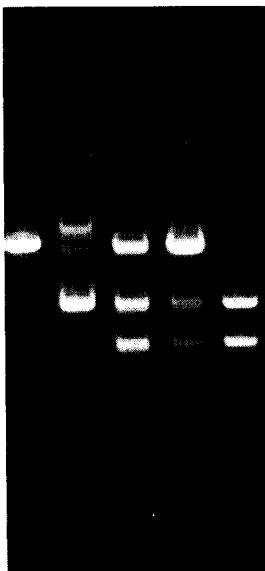


Fig. 10b. Selective hydrolysis of duplex DNA. *Lane 1*, pUC19 linearized with *Hind* III; *lane 2*, pUC 19; *lane 3*, pUC 19 treated with a K116C-containing hybrid nuclease and *Hind* III; *lane 4*, pUC19 treated with a Y113A, K116C hybrid nuclease and *Hind* III; *lane 5*, pUC 19 treated with *Bgl* I

mains to be done in order to determine the sequence and structural requirements for D-loop formation with the hybrid enzymes. Nevertheless, this work is an important step toward the development of strategies for selective cleavage of large linear chromosomal DNAs.

4.12 Sequence-Selective Cleavage of RNA

As noted earlier, studies of RNA structure and function have been hampered by the failure to isolate an analogous class of RNA restriction enzymes from nature. To overcome this obstacle, strategies are being developed for site-selectively hydrolyzing RNAs at specific sites [44–46].

Because staphylococcal nuclease hydrolyzes RNA, it seemed likely that hybrid oligonucleotide-directed nucleases would be able to sequence-specifically cleave RNA. The first substrates to be assayed were unstructured (60–65 bases) RNA's that were prepared by runoff transcription [90, 91]. Hybrid nucleases were constructed using the K116C mutant nuclease and 15 nt oligonucleotide binding sites. As was the case with DNA substrates, the oligonucleotide binding domain selectively delivered the hydrolytic activity of staphylococcal nuclease to a 3 to 5 nucleotide region directly adjacent to the hybridization site [92]. The multiple cleavage sites suggest that there is considerable conformational freedom in the RNA substrate. Interestingly, selective cleavage by the K116C hybrid nuclease occurred even without prior hybridization of the oligonucleotide–nuclease adduct to the target site suggesting that the rate of hybridiz-

ation is more rapid than that of nonselective cleavage. Greater than 90% selective cleavage of RNA could be achieved with an excess of enzyme relative to substrate.

4.13 Sequence-Selective Cleavage of Structured RNA Substrates

The efficient cleavage of short RNA substrates by the K116C hybrid nuclease suggested that more structured, naturally occurring RNAs could also be selectively hydrolyzed [82]. Cleavage reactions were carried out on M1 RNA [93], the catalytic subunit of *E. coli*. RNase P (377 bases) and 16S RNA [94], a 1520 base component of the ribosome 30S subunit. Cleavage reactions were carried out by prior hybridization of the hybrid enzyme to the target RNA at 65°C (in the absence of Ca^{2+}), conditions under which much of the RNA secondary and tertiary structure can be melted out. Since staphylococcal nuclease is rather stable to thermal denaturation [62–65], oligonucleotide binding sites with melting temperatures (20–22 mers) of $\sim 65^\circ\text{C}$ were used to deliver the nuclease to the target sequence. Longer reaction times (20 s) were required with these structured RNAs. M1 RNA was cleaved primarily at one

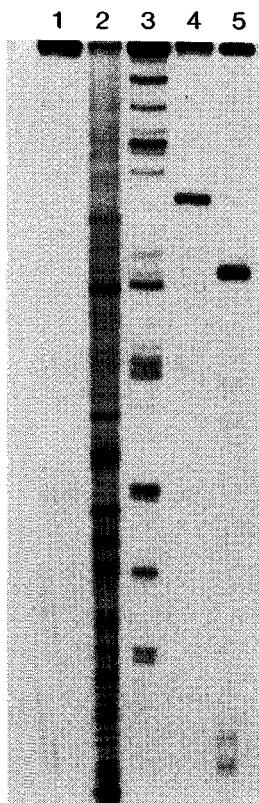
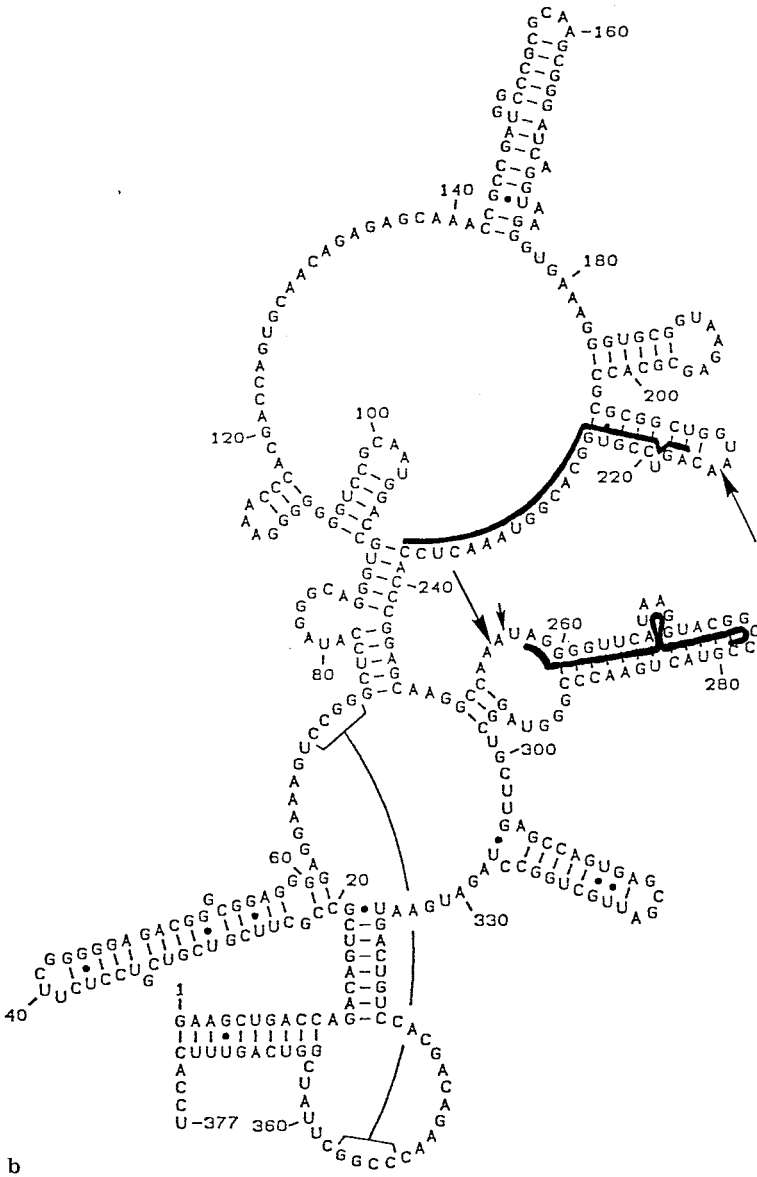


Fig. 11a. Site-selective cleavage of M1 RNA. Lane 1, undigested M1 RNA; Lane 2, a hydroxide ion cleavage ladder, lane 3, digestion of M1 RNA by K116C staphylococcal nuclease; lane 4, digestion of 5'-end labeled M1 RNA by a hybrid nuclease; lane 5, digestion of 3'-end labeled M1 RNA by a hybrid nuclease



b

Fig. 11b. Secondary structure map of M1 RNA, showing the results of two separate hybrid nuclease digestions. The *black lines* denote the 22 nt hybrid nuclease target sequences, and the *arrows* indicate the sites of cleavage; A²¹⁴pA²¹⁵ and A²⁵⁵pA²⁵⁶

position, A²¹⁴ pA²¹⁵, in a hairpin loop by a 22-nt hybrid enzyme that binds largely to a putative single-stranded region of M1 RNA [82]. A second hybrid nuclease (22 nt) that binds a putative duplex region of M1 RNA also cleaved the RNA primarily at one position, A²⁵⁵ pA²⁵⁶. Under optimal conditions, M1

RNA was digested with one equivalent of hybrid enzyme to yield 50% site-selective product and 20% nonselective products. Product yields could be improved by annealing the hybrid nuclease to RNA, carrying out the cleavage reaction, quenching with EGTA, reannealing, and carrying out the reaction again. *E. coli* 16S RNA was cleaved with high selectivity using a 22-nt hybrid nuclease, although the cleavage site could not be determined to nucleotide resolution because of the large size of the cleavage fragments.

Specific cleavage of RNA by the hybrid nucleases occurs with a variety of secondary structures; the primary requirement for cleavage is an A + U-rich cleavage site. Hybridization at temperatures lower than 45 °C did not result in selective cleavage. Cleavage of M1 RNA with an 11-nt hybrid nuclease gave site-selective cleavage, but in lower yields and with more nonselective hydrolysis relative to the corresponding 22-nt hybrid enzyme. These results suggest that the yield of selective cleavage product depends on the stability of the DNA-RNA hybrid relative to the stability of the local substrate RNA structure. The high selectivity of the K116C hybrid enzyme with these natural RNAs most likely results from a combination of the high specificity of DNA-RNA hybridization and the inherent specificity of the nuclease (which prefers A + U-rich regions). As was the case with single-stranded DNA cleavage, the position of the primary cleavage site relative to the hybridization site of the enzyme typically occurs 2 or 3 bases away from the 5'-terminal A of the target sequence [82].

Cleavage of RNA by the hybrid nuclease is relatively sensitive to reaction conditions. Highest specificity is obtained at lower pH values, corresponding to decreased values of k_{cat}/K_m for native staphylococcal nuclease. This result is consistent with the fact that hybrid nucleases constructed from mutants with decreased k_{cat}/K_m values cleave single-stranded DNA substrates with higher specificity than the K116C hybrid nuclease [82].

4.14 Enzymatic Manipulation of the Fragments from Selective RNA Hydrolysis

The utility of hybrid enzymes for the sequence-specific manipulation of RNA was demonstrated by the site-selective cleavage of a tRNA in the anticodon loop, followed by gel purification and ligation of the two half molecules to regenerate the full-length tRNA. Hydrolysis by the nuclease leaves 3' phosphate and 5' hydroxyl termini in contrast to oxidative nucleic acid cleavage strategies. Consequently, addition of T4 polynucleotide kinase and T4 RNA ligase leads to efficient religation of tRNA cleaved by the hybrid nuclease.

5 Oligonucleotide-Directed RNase A

The enzyme ribonuclease A (RNase A) [95] has also been crosslinked to oligonucleotides to afford hybrid enzymes which can selectively hydrolyze RNA. In this case, autolysis of the oligodeoxyribonucleotide binding site cannot occur.

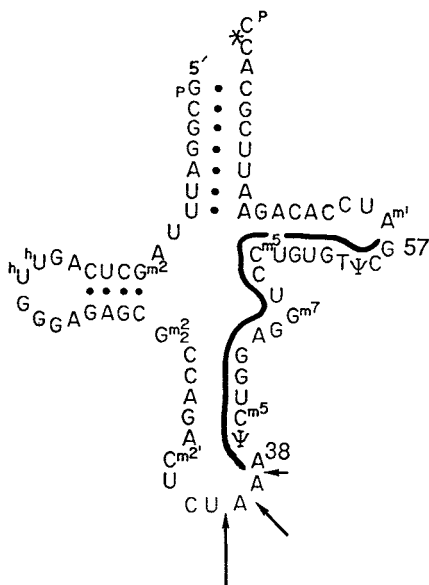


Fig. 12. Sequence of the tRNA; the *black line* denotes the 20 base target sequence, and the *arrows* indicate the sites of cleavage by the hybrid nuclease

RNase A is again an ideal enzyme for semisynthetic studies. The enzyme is small (consisting of 124 amino acids), has been extensively studied by chemical and physical methods [95] and is stable to heat, low pH and most denaturing organic solvents such as phenol or glycerol [95].

The enzyme cleaves RNA with low specificity, cleaving preferentially to the 3' side of pyrimidines, although cleavage also occurs to the 3' side of purines to a lesser extent. The k_{cat}/K_m for the hydrolysis of CpC is $\sim 10^7-10^8 \text{ M}^{-1} \text{ sec}^{-1}$, approaching the diffusion controlled limit [96].

The crystal structure of bovine pancreatic ribonuclease has been investigated by a number of groups. Diffraction data to 2 Å has been reported by Wlodawer [97]. The catalytic mechanism of RNA hydrolysis by RNase A has not been fully defined, but most likely involves activation of the ribose 2'-OH by histidine 12 (or 119) to facilitate the in-line addition of the 2'-hydroxyl to the phosphodiester [98, 99]. Protonated histidine 119 (or 12) may assist in activating the 5'-OH as a leaving group while lysine 41 acts to increase the electrophilicity of the phosphorus and stabilize a trigonal-bipyramidal intermediate. Hydrolysis of the 2',3'-cyclic phosphate would then involve the reverse reaction with H_2O acting as the nucleophile.

Based on the chemical, physical and structural information available on ribonuclease A, attachment of an oligodeoxyribonucleotide of defined length and sequence to the α -amino group of lysine 1 of RNase A should align a hybridized RNA with the active site residues of the enzyme. Because RNase A contains four disulfide bridges, we initially chose to introduce the thiol into fully folded, active RNase A by chemical methods.



Fig. 13. Crystal structure of RNase S.

5.1 RNase S Hybrid Nucleases

Limited digestion of RNase A by subtilisin produces an enzymatically active ribonuclease termed RNase S. RNase S can be separated into S-peptide (amino acids 1–20) and the S-protein (amino acids 21–124), neither of which show catalytic activity [99]. When mixed together stoichiometrically at 25 °C, pH 7.0, in the presence or absence of substrate, a noncovalent complex ($K_d = 1 \times 10^{-5} \text{M}$) is formed with activity similar to native enzyme [101]. This property of RNase A has enabled a variety of analogues of RNase S to be synthesized with amino acid substitutions (natural and synthetic) in positions 1–20 [102, 103].

An S-peptide fragment containing a cysteine at position 1 was therefore synthesized by solid-phase Merrifield synthesis and coupled via a disulfide exchange reaction to a 14 nt 3'-S-thiopyridyl oligonucleotide [104]. The adduct was isolated by anion exchange chromatography and recombined with natural S-protein to generate the hybrid enzyme which was purified by anion exchange chromatography.

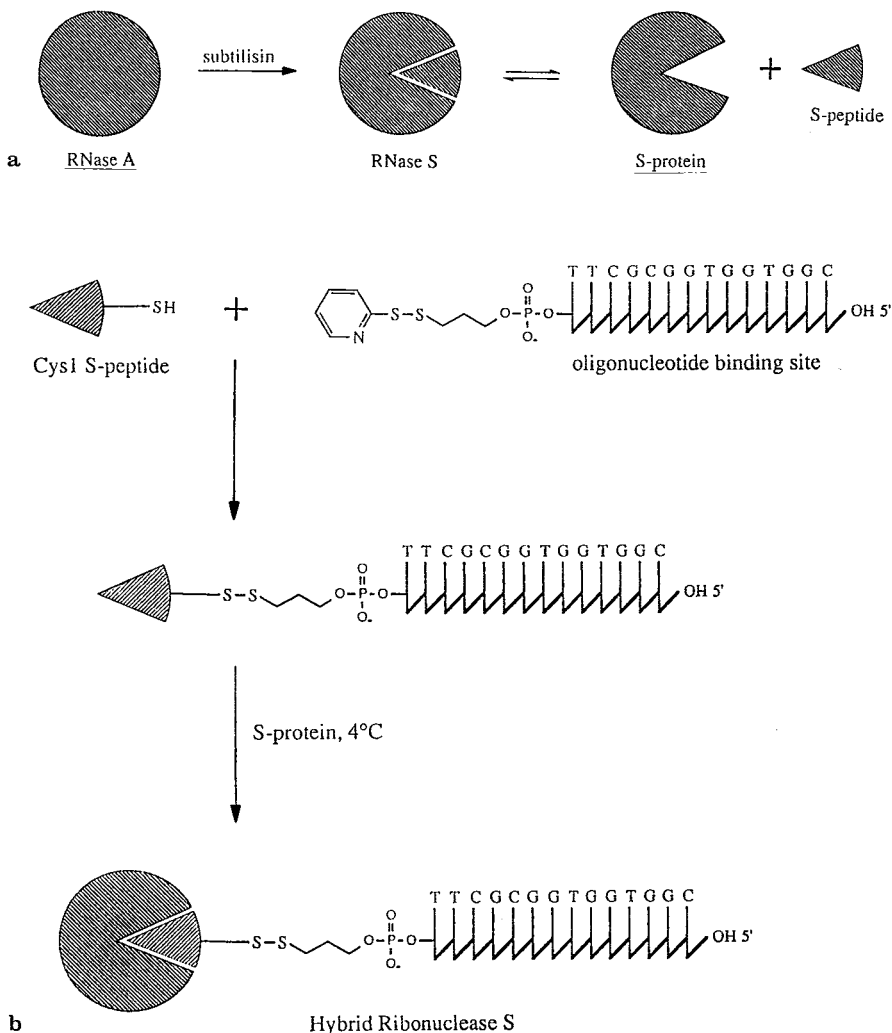


Fig. 14a,b. Construction of hybrid RNase S. (a) RNase S is obtained from a partial subtilisin digestion of RNase A. (b) Crosslinking of K1C S-peptide to a 3'-S-thiopyridyl oligonucleotide

The ability of the hybrid enzyme to site-selectively cleave RNA was assayed with a 62-nucleotide single-stranded RNA prepared by runoff transcription [90, 91]. The RNase S hybrid nuclease cleaved the 62 nt unstructured RNA substrate with high selectivity when cleavage was carried out at temperatures below 10 °C. Unfortunately, hybridization and cleavage at elevated temperatures (>37 °C) led to diminished cleavage efficiency and specificity, most likely due to dissociation of the S-protein from the peptide-oligonucleotide adduct. Cleavage occurred primarily at one pyrimidine-purine site adjacent to the site of oligonucleotide

hybridization. Again the high selectivity is likely a result of combining the specificity of the oligonucleotide with the inherent specificity of RNase S. Addition of S-protein to a preformed S-peptide-oligonucleotide RNA complex did not afford selective cleavage.

5.2 RNase A Hybrid Nuclease

The gene for RNase A has been cloned and expressed [105], allowing the introduction of the K1C mutation via site directed mutagenesis. This mutant retains RNase activity, indicating that it folds properly despite the introduction of a ninth cysteine. Direct fusion of the oligonucleotide binding site to RNase A via cysteine 1 generated a hybrid nuclease which specifically cleaved unstructured RNA substrates. Moreover the adduct was thermally stable, allowing it to be used at elevated temperatures [106].

However, the K1C RNase A-containing hybrid nuclease selectively hydrolyses RNA only under a narrow range of conditions. Another mutant RNase A, K41R, has a k_{cat} 1% that of wild type enzyme while the K_m is unchanged [105]. It will be of interest to construct and assay a K1C, K41R RNase A hybrid nuclease and determine if, like the Y113A, K116C staphylococcal nuclease, it too will hydrolyze substrate with improved specificity.

6 Conclusion

Hybrid nucleases have been generated that are capable of sequence-specifically hydrolyzing large natural RNAs, single-stranded DNAs, and duplex DNAs. These enzymes can function catalytically and their specificity approaches that of restriction enzymes. This work provides powerful new methods for selectively cleaving single-stranded DNA and RNA and is an important step toward the generation of enzymes capable of site-specifically cleaving large chromosomal DNA's.

The fact that one can engineer a high degree of selectivity into nonspecific hydrolytic enzymes illustrates the power of a semisynthetic approach that *combines chemical modification of enzymes with site-directed mutagenesis*. Application of this strategy to peptidases and glycosidases may lead to a new class of selective catalysts for the selective cleavage and ligation of proteins and sugars.

7 References

1. Sharpless KB, Woodward SS, Finn MG (1984) In: Bartmann W, Trost BM (eds) *Selectivity-A goal for synthetic efficiency*, Verlag Chemie, Weinheim, Federal Republic of Germany, 377
2. Noyori R, Ohkuna T, Kitamura M, Takaya H, Sayo N, Kumobayashi H, Akutagawa S (1987) *J Am Chem Soc*, 109: 5856
3. Rebek J (1987) *Science* 235: 78

4. Lehn J (1988) *Angew Chem Int Ed*, 27: 90
5. Breslow R, (1986) *Adv Enzymol*, 58: 1
6. Knowles JR (1987) *Science* 236: 1252
7. Wells JA, Cunningham BC, Graycar TP, Estell DA (1987) *Proc Natl Acad Sci*, 84: 5167
8. Cronin CN, Malcolm BA, Kirsch JF (1987) *J Am Chem Soc*, 109: 2222
9. Noren CJ, Anthony-Cahill SJ, Griffith MC, Schultz PG (1989) *Science*, 244: 182
10. Youderian P, Vershon A, Bouvier S, Sauer RT, Susskind MM (1983) *Cell*, 35: 777
11. Masazumi M, Aiba S (1985) *J Biol Chem*, 260: 15298
12. Das G, Hickey DR, McLendon D, McLendon G, Sherman F (1989) *Proc Natl Acad Sci USA*, 86: 496
13. Liao H, Mckenzie T, Hageman R (1986) *Proc Natl Acad Sci USA*, 83: 576
14. Regan L, DeGrado WF (1988) *Science*, 241: 976
15. Kim PS (1988) *Protein Engineering* 2: 249
16. DeGrado WF, Wasserman ZR, Lear JD (1989) *Science*, 243: 622
17. Richardson JS, Richardson DC (1989) *Trends Biochem Sci*, 14: 304
18. Pollack SJ, Jacobs JW, Schultz PG (1986) *Science*, 234: 1570
19. Tramontano A, Janda KD, Lerner RA (1986) *Science*, 234: 1566
20. Iverson BL, Lerner RA (1989) *Science*, 243: 1184
21. Cochran AG, Sugasawara R, Schultz PG (1988) *J Am Chem Soc*, 110: 7888
22. Shokat KM, Leumann CJ, Sugasawara R, Schultz PG (1989) *Nature*, 338: 269
23. Jackson DY, Jacobs JW, Sugasawara R, Reich SH, Bartlett PA, Schultz PG (1988) *J Am Chem Soc*, 110: 4841
24. Hilvert D, Carpenter SH, Nared KD, Auditor M-T.M (1988) *Proc Natl Acad Sci USA*, 85: 4953
25. Neet KE, Koshland DE (1966) *Proc Natl Acad USA*, 56: 1606
26. Polgar L, Bender ML (1966) *J Am Chem Soc*, 88: 3153
27. Neet KE, Nanci A, Koshland DE (1968) *J Biol Chem*, 243: 6392
28. Polgar L, Bender ML (1970) *Adv Enzymol*, 33: 381
29. Yokosawa H, Ojima S, Ishii J (1977) *J Biochem (Tokyo)*, 82: 869
30. Clark PI, Lowe G (1978) *Eur J Biochem*, 84: 293
31. Nakatsuka T, Sasaki T, Kaiser ET (1987) *J Am Chem Soc*, 109: 3808
32. Wong C-H (1989) *Science*, 244: 1145
33. (a) Kaiser ET, Lawrence DS (1984) *Science*, 226: 505
(b) Radziejewski C, Hilvert D, Kaiser ET (1985) *Biocatalysts in organic syntheses*, Amsterdam, p 81
34. Pollack SJ, Nakayama GR, Schultz PG (1988) *Science*, 242: 1038
35. Pollack SJ, Schultz PG (1989) *J Am Chem Soc*, 111: 1929
36. Vitetta ES, Krolick KA, Miyama-Inaba M, Cushley W, Uhr JW (1983) *Science*, 219: 644
37. Bode C, Matsuoda GR, Hui HY, Haber E (1985) *Science*, 229: 765
38. Haber E, Quartermous T, Matsuoda GR, Runge MS (1989) *Science*, 243: 51
39. Westerberg DA, Carney PL, Rogers PE, Kline SJ, Johnson DK (1989) *J Med Chem* 32: 236
40. Ji TH (1983) *Methods in Enzymol*, 91: 580
41. Brennan M, Davidson PF, Paulus H (1985) *Science*, 229: 81
42. McClelland M, Jones R, Patel Y, Nelson M (1987) *Nucleic Acids Res*, 15: 5985
43. Kim SC, Podhajaska AJ, Syzblski W (1988) *Science*, 240: 504
44. Crouch RJ, Dirksen ML (1982) In: Linn SN, Roberts RJ (eds) *Nucleases Vol 14*, Cold Spring Harbor Press, p 211
45. Shibahara S, Mukai S, Nishihara T, Inoue H, Ohtsuka E, Morisawa H (1987) *Nucl Acids Res* 15: 4403
46. Cech TR, Bass BL (1986) *Ann Rev Biochem* 55: 599
47. Noller H (1984) *Ann Rev Biochem* 53: 119
48. Cimino GD, Gamper HB, Issacs ST, Hearst JE (1985) *Ann Rev Biochem* 54: 1151
49. Dervan PB (1986) *Science*, 232: 464
50. (a) Dreyer GB, Dervan PB (1985) *Proc Natl Acad Sci USA*, 82: 968; (b) Chu BCF, Orgel LE, *ibid*, 963, (c) Chen CB, Sigman DS (1988) *J Am Chem Soc*, 110: 6570
51. Moser HE, Dervan PB (1987) *Science*, 238: 645
52. Stroebel SA, Moser HE, Dervan PB (1988) *J Am Chem Soc*, 110: 7927
53. Mack DP, Iverson BL, Dervan PB (1988) *J Am Chem Soc*, 110: 7572
54. Chen BC, Sigman DS (1987) *Science*, 237: 1197

55. Wallace RB, Shaffer J, Murphy RF, Bonner J, Hirose T, Itakura K (1979) *Nucleic Acids Res*, 6: 3543
56. Li P, Medon PP, Skingle DC, Lanser JA, Symons RH (1987) *Nucleic Acids Res*, 15: 5275
57. Sanger F, Nicklen S, Coulson AR (1977) *Proc Natl Acad Sci USA*, 24: 5463
58. Zoller M, Smith M (1983) *Methods Enzymol*, 100: 468
59. Green PJ, Pines O, Inouye M (1986) *Ann Rev Biochem*, 55: 569
60. Zuckermann R, Corey DR, Schultz PG (1987) *Nucleic Acid Res*, 15: 5305
61. Corey DR, Schultz PG, unpublished results
62. Tucker PW, Hazen EE, Cotton FA (1978) *Mol Cell Biochem*, 22: 67
63. Tucker PW, Cotton FA, Hazen EE (1979) *Mol Cell Biochem*, 23: 3
64. Tucker PW, Cotton FA, Hazen EE (1979) *Mol Cell Biochem*, 23:
65. Tucker PW, Hazen EE, Cotton FA (1979) *Mol Cell Biochem*, 23: 131
66. (a) Serpersu EH, Shortle D, Mildvan AS (1987) *Biochemistry*, 26: 1289; (b) *Ibid* (1986) 25: 68
67. Cuatrecasas P, Fuchs S, Anfinsen C (1967) *J Biol Chem*, 242: 1541
68. Cotton FA, Hazen EE, Legg MJ (1979) *Proc Natl Acad Sci USA* 76: 2551
69. Takahara M, Hibler DW, Barr PJ, Gerlt JA, Inouye M (1985) *J Biol Chem* 260: 2670
70. Shortle D (1986) *J Cell Biochem*, 30: 281
71. Alber T, Sun DB, Wilson K, Wozniak JA, Cook SP, Matthews BW (1987) *Nature*, 330: 41
72. Pery LJ, Wetzel R (1986) *Biochemistry*, 25: 733
73. Falke JJ, Koshland DE (1987) *Science*, 237: 1596
74. Matsumura M, Matthews BW (1989) *Science*, 243: 792
75. Corey DR, Schultz PG (1989) *J Biol Chem*, 264: 3666
76. Kunkel TA (1985) *Proc Natl Acad Sci*, 82: 488
77. Corey DR, Schultz PG (1987) *Science*, 238: 1401
78. Wang D (1979) *Biochemistry*, 18: 4449
79. Glazer AN, DeLang RJ, Sigman DS *Chemical Modification of Proteins*, North Holland/Elsevier, Amsterdam, 1975
80. Corey DR, Pei D, Schultz PG (1989) *Biochemistry*, in Press
81. Messing J (1983) *Methods in Enzymol*, 101: 20
82. Zuckermann RN, Schultz PG (1989) *Proc Natl Acad Sci USA*, 86: 1766
83. Wiegand RC, Beattie KL, Holloman WK, Radding CM (1977) *J Mol Biol*, 116: 805
84. Landgren U, Kaiser R, Sanders J, Hood L (1988) *Science*, 241: 1077
85. Wang Y (1988) *Biotechniques*, 6: 843
86. Cheng S, Van Houten B, Gamper HB, Sancar A, Hearst JE (1988) *J Biol Chem* 263: 15110
87. Pugh BF, Cox MM (1988) *J Mol Biol* 203: 479
88. Corey DR, Pei D, Schultz PG (1989) *J Am Chem Soc*, in Press
89. Yanisch-Perron C, Viere J, Messing J (1985) *Gene*, 33: 103
90. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC (1987) *Nucleic Acids Res*, 15: 8783
91. Sampson JR, Uhlenbeck OC (1988) *Proc Natl Acad Sci USA*, 85: 1033
92. Zuckermann RN, Corey DR, Schultz PG (1988) *J Am Chem Soc*, 110: 1614
93. James BD, Olsen GJ, Lieu J, Pace NR (1988) *Cell*, 52: 19
94. Stern S, Weiser B, Noller HE (1988) *J Mol Biol*, 204: 447
95. Blackburn P, Moore S (1982) *The Enzymes*, 3rd edition, 15: 317
96. Erman JE, Hammes GGJ (1966) *Am Chem Soc*, 88: 5614
97. Wlodawer A, Bott R, Sjolín L (1982) *J Biol Chem*, 257: 1325
98. Witzel H, Barnard EA (1962) *Biochem Biophys Res Comm*, 7: 289
99. Brocklehurst K, Crook EM, Wharton CW (1967) *Chem Comm*, 63: 66
100. Richards FM, Vithayathil PJ (1959) *J Biol Chem*, 234: 1459
101. Finn FM (1972) *Biochemistry*, 11: 1474
102. Walder RY, Walder JA (1988) *Proc Natl Acad Sci USA*, 85: 5011
103. Chaikin IM (1981) *CRC Crit Rev Biochem*, 11: 255
104. Zuckermann RN, Schultz PG (1988) *J Am Chem Soc*, 110: 6592
105. Raines RT, Rutter WJ, Manuscript in preparation
106. Zuckermann R, Schultz PG, Raines RT, Rutter WJ. Manuscript in preparation

Note added in proof: It has been recently shown that triple helix formation and protein-DNA complex formation can be used to target semisynthetic nucleases selectively to duplex DNA.

