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5 May 1987; accepted 10 September 1987

Generation of a Hybrid Sequence-Specific Single-Stranded Deoxyribonuclease

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The relatively nonspecific single-stranded deoxyribonuclease, staphylococcal nuclease, was selectively fused to an oligonucleotide binding site of defined sequence to generate a hybrid enzyme. A cysteine was substituted for Lys^{116} in the enzyme by oligonucleotide-directed mutagenesis and coupled to an oligonucleotide that contained a 3′-thiol. The resulting hybrid enzyme cleaved single-stranded DNA at sites adjacent to the oligonucleotide binding site.

ONE APPROACH TO DEVELOPING biological catalysts with tailored specificities involves redesigning existing enzyme active sites by the method of oligonucleotide-directed mutagenesis (*1*). Alternatively, entire binding or catalytic domains may be added or replaced to generate hybrid enzymes with novel specificities. We have applied this latter strategy to the construction of a sequence-specific single-stranded deoxyribonuclease (DNase) consisting of the relatively nonspecific enzyme, staphylococcal nuclease, selectively fused to an oligonucleotide binding site of defined sequence. The hybrid enzyme selectively cleaves single-stranded DNA adjacent to the oligonucleotide binding site. The hybrid hydrolyzes the phosphodiester bond of DNA (*2*), in contrast to existing chemical strategies for selectively cleaving DNA that result in degradation of the ribose backbone (*3–5*).

Staphylococcal nuclease is a well-characterized stable enzyme consisting of a single polypeptide chain 149 amino acids in length (*6–9*). The enzyme preferentially hydrolyzes the phosphodiester bonds of single-stranded RNA, single-stranded DNA, and duplex DNA at A,U- or A,T-rich regions to generate 3′-phosphate and 5′-hydroxyl termini (*10*); Ca^{2+} is required for enzymatic activity, providing a mechanism for modulating enzyme action (*6*). The structure and mechanism of staphylococcal nuclease have been elucidated from a series of chemical, physical, and genetic studies. An x-ray crystal structure of a staphylococcal nuclease–diphosphothymidine (pTp)–Ca^{2+} complex has been determined to 1.5 Å (Fig. 1) (*8, 11*). The pyrimidine ring of the inhibitor pTp fits into a hydrophobic pocket at the enzyme surface and the 5′-phosphate is near Arg^{35} and Arg^{87}. Glu^{43} is thought to act as a general base for activation of the attacking water molecule, whereas Arg^{35}, Arg^{87}, and Ca^{2+} stabilize the trigonal bipyramidal transition state (*11, 12*).

The geometry of the bound inhibitor pTp suggests that coupling of the 3′-terminus of an oligonucleotide binding domain to Lys^{116} on the enzyme surface should align a hybridized RNA or DNA substrate with the enzyme active site. A flexible tether was incorporated to allow some variability between the hybridized substrate and the active site residues. An oligomer 15 nucleotides (nt) in length, 5′-CCCGCACAAGCCG-CT-3′ (melting temperature, 62°C) (*13*), was coupled to staphylococcal nuclease by a disulfide exchange reaction. This coupling strategy required the introduction of a free thiol at both the 3′-terminus of the oligonucleotide and at residue 116 of the enzyme. Subsequent disulfide exchange should proceed under mild conditions and with high selectivity because of the absence of competing free thiols in DNA and staphylococcal nuclease.

We have adapted existing methods for the solid-phase phosphotriester or phosphoramidite synthesis of oligonucleotides to allow rapid incorporation of free 3′-thiols (*14*). The 3′-thiol is introduced in the form of a disulfide linkage between the 3′-nucleoside and the solid-phase support prior to the first step of oligonucleotide synthesis (Fig. 2). After synthesis and deprotection of the oligomer, the disulfide bond is cleaved and subsequently reacted with 2,2′-dithiodipyridine to afford the 3′-*S*-thiopyridyl oligonucleotide. This modified oligonucleotide has been demonstrated to selectively react in high yields under mild conditions with a thiol-containing fluorescent probe (*14*).

A free thiol was introduced into staphylococcal nuclease by replacement of Lys^{116}

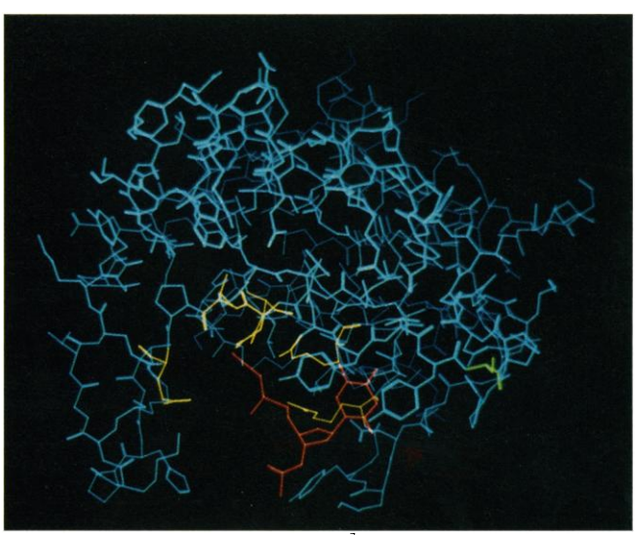

Fig. 1. X-ray crystal of a staphylococcal nuclease–pTp–Ca^{2+} complex (*11*). The active site residues Asp^{21}, Arg^{35}, Asp^{40}, Thr^{41}, Glu^{43}, and Arg^{87} are yellow, the inhibitor pTp is red, and Cys^{116} is green.

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with Cys^{116} via oligonucleotide-directed mutagenesis. This approach was adopted since there are no other cysteine residues present in the mutant enzyme (*15, 16*) and the gene that encodes staphylococcal nuclease has been cloned and overproduced as a fusion protein with the omp A protein leader sequence (*16, 17*). Gel electrophoresis of the purified Cys^{116} mutant (*18–23*) without prior reduction of the enzyme revealed that the isolated enzyme was dimeric. Reduction of the dimer with dithiothreitol afforded monomeric enzyme that eluted with a shorter retention time from a Pharmacia Mono S cation exchange column and comigrated with native staphylococcal nuclease on a sodium dodecyl sulfate polyacrylamide gel (*24*). The activity of the Cys^{116} mutant enzyme was determined (*12*) in the presence of 0.05*M* dithiothreitol in order to maintain the Cys^{116} enzyme in the monomeric state. The Michaelis constants, k_{cat} and K_m, of the monomeric Cys^{116} mutant are 3.2 ± 0.5 sec^{-1} and 8.6 ± 2.5 μg/ml, respectively, similar to those reported for the native enzyme (*12*). Thiol titrations of the monomeric enzyme with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) afforded a stoichiometry of 1.0 free thiol per monomer, which confirmed the Lys^{116} to Cys^{116} mutation (*25*).

The monomeric Cys^{116} mutant enzyme was cross-linked to the 3′-*S*-thiopyridyl oligonucleotide to produce the hybrid enzyme (Fig. 3). The adduct has a shorter retention time relative to the Cys^{116} mutant on a Pharmacia Mono S cation exchange column, which is consistent with the anionic nature of the adduct. The adduct is stable for months at 0°C in the presence of the Ca^{2+} chelator EGTA (*6*). Addition of 0.05*M* dithiothreitol to the oligonucleotide-nuclease adduct resulted in the disappearance of the adduct peak and the reappearance of peaks that comigrated (Mono S) with the 3′-thiol–oligonucleotide and Cys^{116} mutant monomer. The adduct had the characteristic λ_{max} of 260 nm associated with oligonucleotides and the phosphodiesterase activity associated with staphylococcal nuclease.

The specificity of the staphylococcal nuclease–oligonucleotide adduct was assayed with a ^{32}P 5′-end-labeled (*26*) 64-nt single-stranded DNA fragment by electrophoresis on a high-resolution denaturing polyacrylamide gel (*27*) (Fig. 4). Inspection of the histogram of the cleavage pattern produced by the hybrid enzyme (Fig. 5) reveals that the oligonucleotide binding domain selectively delivers the hydrolytic activity of staphylococcal nuclease to single-stranded DNA. 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. Greater than 90% of the substrate can be selectively converted to product at a hybrid enzyme to DNA ratio of 2 to 1 (*28*). Cleavage by the hybrid enzyme occurs primarily at one site (Fig. 4), and to a lesser extent at the surrounding two to three nucleotides. The presence of minor cleavage sites likely results from the flexibility of the six methylene spacer that links the 3′-nucleotide of the oligonucleotide to Cys^{116} of the nuclease. Optimization of linker length and flexibility may achieve single-site specificity. Sub-

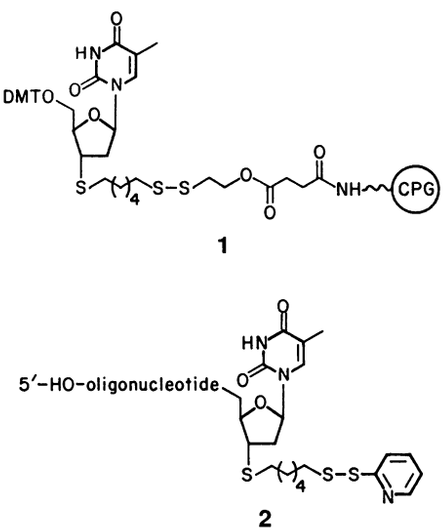

Fig. 2. The 15-nt fragment was synthesized from 3′-thymidine linked to controlled pore glass (CPG) via a disulfide linkage **1**. After synthesis of the oligonucleotide, the protecting groups were removed by standard methods and the product was treated with 0.06*M* dithiothreitol at 37°C. Reversed-phase chromatography yielded the purified 3′-thiol oligonucleotide, which was then directly reacted for 8 hours with 5 m*M* 2,2′-dithiodipyridine in 15% acetonitrile, 100 m*M* sodium phosphate buffer, *p*H 5.5. The resulting adduct **2** was purified by reversed-phase high-pressure liquid chromatography to afford the 3′-*S*-thiopyridyl oligonucleotide.

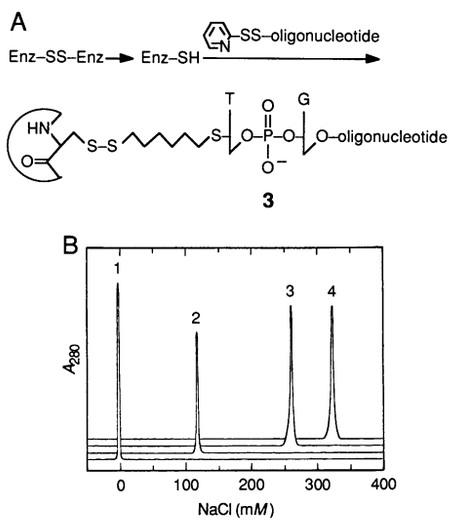

Fig. 3. Formation of the staphylococcal nuclease–oligonucleotide adduct. (**A**) Dimeric enzyme (Enz-SS-Enz; 34 nmol in 1.0 ml of 50 m*M* NaCl, 2 m*M* Hepes, *p*H 6.8) was reduced to the monomer by treatment with 50 m*M* dithiothreitol for 8 hours at 37°C. The monomeric enzyme (Enz-SH) was purified by chromatography on Sephadex G-10 followed by cation exchange chromatography on a 5-cm Pharmacia Mono *S* column with aqueous 2.5 m*M* EGTA, 50 m*M* Hepes, *p*H 7.5, and a gradient of 0 to 0.5*M* NaCl. The purified enzyme (17 nmol in 1 ml of column buffer) was reacted with 4.4 nmol of thiopyridyl-activated oligonucleotide (20 μl; 50 m*M* tris, *p*H 8.0). Formation of the cross-linked adduct **3** was observed spectrophotometrically by monitoring the release of thiopyridyl anion at 343 nm. (**B**) The adduct was purified by cation exchange chromatography as described above in an overall yield of 60%. Peak 1 is the 15-nt 3′-*S*-thiopyridyl oligonucleotide 5′-CCCGCACAAGCCCCT-3′; peak 2 is the oligonucleotide–staphylococcal nuclease adduct; peak 3 is monomeric mutant Cys^{116} staphylococcal nuclease; and peak 4 is dimeric enzyme.

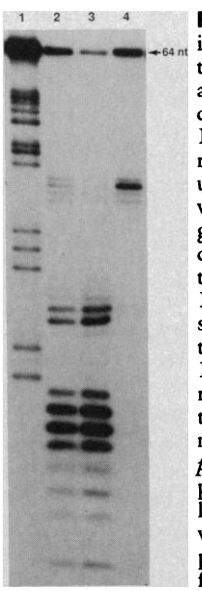

Fig. 4. A 15% polyacrylamide denaturing gel showing the sequence specific cleavage; the 64-nt fragment is denoted by the arrow. Lane 1 is the Maxam-Gilbert G reaction. Lane 4 is the product of a cleavage reaction with 11 n*M* cross-linked oligonucleotide-nuclease adduct. Lanes 2 and 3 show the background cleavage by 100 n*M* mutant Cys^{116} staphylococcal nuclease, in the presence and absence of 11 n*M* 15-nt binding site, respectively. Cleavage reactions were performed in 25 m*M* Hepes, 70 m*M* NaCl, *p*H 7.5; all reactions were performed at 5°C with 4 n*M* labeled DNA in a reaction volume of 12 μl. The samples were annealed at 65°C for 10 minutes. After cooling, cleavage was initiated through the addition of 2 μl of a 25 m*M* Ca^{2+} solution and was quenched after 1 second by the addition of 20 μl of formamide that contained 10 m*M* 3′,5′-diphosphothymidine.

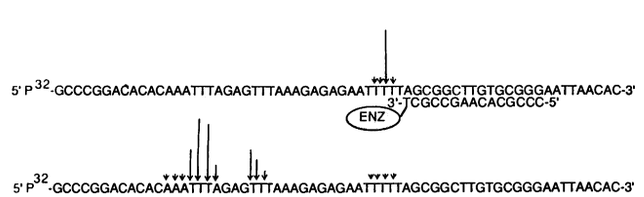

Fig. 5. Histograms of DNA cleavage patterns in Fig. 4 (lanes 2 and 4). The heights of the arrows show the relative cleavage intensities at the indicated bases.

strates with one thymidine adjacent to the oligonucleotide binding site should afford cleavage of a single bond.

Termination of the cleavage reaction at short reaction times resulted in highly selective hydrolysis, whereas longer reaction times caused an increasing amount of nonselective secondary cleavage of the substrate DNA. Nonselective cleavage may arise from autocleavage of the oligonucleotide binding domain, a problem that may be overcome with a nonhydrolyzable binding site. Cleavage of the substrate without prior hybridization also resulted in selective cleavage, suggesting that nonselective cleavage by the hybrid enzyme is slow. The addition of 1 μg of poly(dA) DNA to the reaction mixture decreased nonselective cleavage of the substrate DNA. In conclusion, we have demonstrated that selective introduction of an oligonucleotide binding site into the relatively nonselective DNase staphylococcal nuclease generates a hybrid sequence-specific DNase. It may be possible to rationally alter the specificity of other enzymes by a similar strategy.

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29. Supported in part by a Presidential Young Investigator Award from the National Science Foundation (CHE85-43106) and by the Petroleum Research Foundation, administered by the American Chemical Society. We thank J. Gerlt for the gift of plasmid pONF 1.

19 June 1987; accepted 23 October 1987

A Complete Mapping of the Proteins in the Small Ribosomal Subunit of *Escherichia coli*

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The relative positions of the centers of mass of the 21 proteins of the 30*S* ribosomal subunit from *Escherichia coli* have been determined by triangulation using neutron scattering data. The resulting map of the quaternary structure of the small ribosomal subunit is presented, and comparisons are made with structural data from other sources.

THE POLYMERIZATION OF AMINO ACids to form proteins is catalyzed in all organisms by a nucleoprotein enzyme known as the ribosome. Ribosomes are template-directed polymerases whose substrates are aminoacyl–transfer RNAs; messenger RNAs are their templates.

The ribosome of *Escherichia coli* is a typical prokaryotic ribosome, similar in design to its larger, eukaryotic homologs (*1*). It consists of a large, 50*S* subunit and a small, 30*S* subunit. The 50*S* subunit contains 32 different proteins (average molecular weight ~14,500) and two ribosomal RNAs (rRNAs), 5*S* RNA (120 nucleotides) and 23*S* RNA (2904 nucleotides). The 30*S* subunit has 21 proteins (average molecular weight ~16,500) and a single 16*S* rRNA (1541 nucleotides). The proteins of both ribosomal subunits are present in unit stoichiometry with the exception of the 50*S* protein L7-L12, which is present as a tetramer (L7 is an acetylated form of L12) (*2*). The molecular weight of the assembly is 2.3×10^6, exclusive of counterions.

The overall structure of the *E. coli* ribosome has been investigated by image reconstruction methods at low resolution [for example (*3*)], and an atomic resolution, crystallographic structure may ultimately become available (*4*). A large number of methods have been used to probe its quaternary organization: immune–electron microscopy (IEM) (*5, 6*), chemical cross-linking (*7, 8*), fluorescence energy transfer (FET) (*9*), and neutron scattering (*10–12*). Assembly of the ribosome (*13*) and the protein binding sites on the rRNAs (*14*) have also been studied. The completed analysis of the positions of the proteins in the small ribosomal subunit of *E. coli* by neutron scattering is presented here.

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