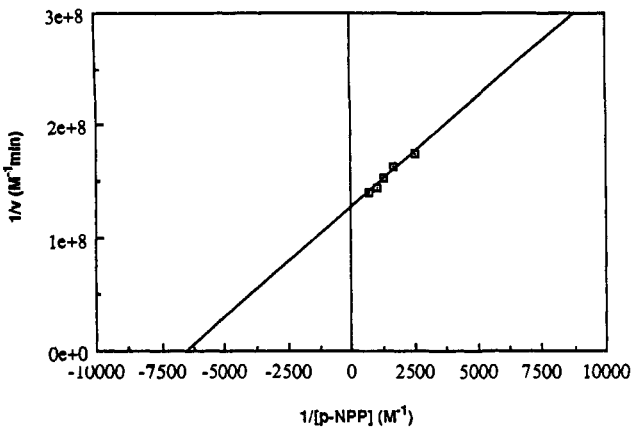

Figure 1. Lineweaver-Burk plot of 38E1-catalyzed hydrolysis of *p*-NPP: [38E1] = 6.7 μM in 50 mM CHES, 25 mM NaCl, pH 9.0; [*p*-NPP] = 1.5, 1.0, 0.8, 0.6, 0.4 mM. Velocity measurements were performed in triplicate at each substrate concentration, monitoring the increasing absorbance at 400 nm corresponding to the formation of *p*-nitrophenolate ion.

to catalyze the hydrolysis of *p*-nitrophenyl phosphate **2** (*p*-NPP). Five of the 20 antibodies were found to catalyze the hydrolysis of the phosphate monoester at a significant rate above the uncatalyzed background reaction. One of these antibodies (38E1) was characterized in more detail.

The *p*-NPP hydrolysis reaction catalyzed by 38E1 displayed a pH optimum at alkaline pH; consequently, all kinetic parameters were measured in the presence of 6.7 μM antibody 38E1 in 50 mM CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid], 25 mM NaCl at pH 9.0.[7] Under these conditions, 38E1 catalyzed the hydrolysis of *p*-NPP in a manner consistent with Michaelis-Menten kinetics.[8] A Lineweaver-Burk plot (Figure 1) of the steady-state data afforded a k_{cat} of 0.0012 min^{-1} and a K_M of 155 μM. The observed first-order rate constant for the uncatalyzed hydrolysis of *p*-NPP in the same buffer system was determined to be 1.5×10^{-7} min^{-1}. Greater than 11 turnovers per antibody molecule were measured with no apparent change in V_{max}, demonstrating that the antibody functions catalytically.

The Fab fragment of 38E1, generated by partial papain digestion,[9] retained the catalytic activity of the intact IgG, supporting the concept that catalysis occurs in the antibody combining site. The antibody 38E1 also retained activity in the presence of the metal chelator EDTA, which abolishes the catalytic activity of the metal-dependent phosphatases.[10] Moreover, several ester- and carbonate-cleaving catalytic antibodies have been generated in our laboratory from haptens containing nitrophenyl phosphonate substructures, and none of these antibodies were found to catalyze the hydrolysis of *p*-NPP.[11]

The antibody-catalyzed reaction was competitively inhibited by hapten **1**. A Dixon analysis[12] with hapten **1** afforded a K_i of 34 μM. The V_{max} value of 1.0×10^{-8} min^{-1} derived from the Dixon plot was in good agreement with the V_{max} value obtained from the Lineweaver-Burk analysis. The substrate specificity of antibody 38E1 was also examined. Phosphate monoesters **3**-**5** were found to be poor substrates for antibody 38E1, with hydrolytic rates barely detectable above the uncatalyzed reaction.[13] This high level of specificity, favoring the para-substituted phenyl phosphate, is consistent with an antibody-catalyzed reaction and most likely derives from the para-substituted aryl ring of hapten **1**. In contrast, alkaline phosphatases from a wide range of sources exhibit low substrate specificity, hydrolyzing positional isomers of aryl phosphates with similar rates.[14]

Mechanistic aspects of this antibody-catalyzed reaction are currently being explored, including both electrostatic catalysis and the notion that hapten **1**, like the less stable vanadate esters, mimics a species involved in attack of water on the phosphate monoester. In fact, it has been reported that 2-hydroxy carboxylic acids and tartrates, which are structural analogues of hapten **1**, act as inhibitors of phosphatase enzymes.[15] Consequently, a more detailed understanding of the structural features of hapten **1** leading to antibody catalysis could provide a new class of selective phosphoryl-transferase enzyme inhibitors.

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(7) All kinetic assays were performed on a Kontron Uvikon 860 spectrophotometer. Reaction volumes of 1.2 mL were used, and time-point measurements were recorded at 400 nm (λ_{max} for *p*-NPP, $\epsilon = 17000$ M^{-1} cm^{-1}) in 1-mL cuvettes. Typically, a 50× concentrated substrate stock solution in water was diluted into a solution of the antibody sample in 50 mM CHES, 25 mM NaCl, pH 9.0 assay buffer. The reactions were incubated at 30 °C, and time points were recorded over a 30-h reaction period. Under these conditions, the hydrolysis reaction proceeded to <5% completion.

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Engineering Protein Specificity: Gene Manipulation with Semisynthetic Nucleases

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During the past decade, site-directed mutagenesis and chemical modification have been extensively used in attempts to engineer proteins with novel functions or altered specificities. For example, mutagenesis has been used to alter the specificities of aspartate aminotransferase, a dehydrogenase, and 434 repressor, and semisynthesis has been used to generate a redox-active flavopapain.[1] By using a combination of *both* chemical and genetic approaches, we were able to convert a relatively nonspecific phosphodiesterase, staphylococcal nuclease, into a molecule capable of sequence-specifically hydrolyzing RNA, single-stranded DNA, and duplex DNA.[2,3] Adducts of staphylococcal nuclease with either oligo-

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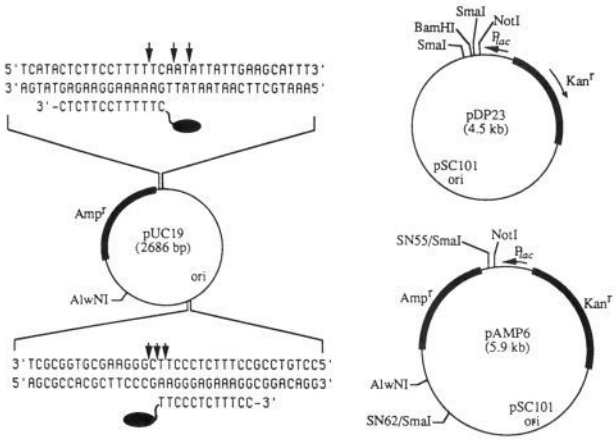

Figure 1. The binding and cleavage sites of the two semisynthetic nucleases in plasmid pUC19, and maps of the plasmids pDP23 and pAMP6. The arrows above the DNA sequences indicate the cleavage sites of SN55 and SN62.

nucleotides or a DNA-binding protein selectively recognize duplex DNA via D-loop formation,[4] triple-helix formation,[5] or specific protein–DNA contacts.[6] In order to demonstrate the extent to which protein specificity can be rationally altered by the above approach, we asked whether semisynthetic nucleases could be constructed which, in lieu of restriction enzymes, can be used to selectively isolate and transfer genetic material. We now describe the use of semisynthetic nucleases, consisting of staphylococcal nuclease linked to the 5′ termini of homopyrimidine oligonucleotides, to substitute the natural promoter for ampicillin resistance gene with the *lac* transcriptional promoter.

Two semisynthetic nucleases, SN55 and SN62, were synthesized[3] by coupling 13-nucleotide (13-nt) and 12-nt pyrimidine oligonucleotides, 5′-$CT_5C_2T_2CTC$-3′ and 5′-$T_2C_3TCT_3C_2$-3′, respectively, to a K84C staphylococcal nuclease mutant. Nuclease SN55 was expected to bind to the sequence at positions 2488–2500 in the plasmid pUC19[7] via triple-helix formation and to cleave between the ribosomal binding site (RBS) and −10 region of the promoter sequence upstream of the β-lactamase gene (Figure 1).[8] The second nuclease, SN62, was expected to bind and cleave the sequence at positions 1028–1039 (Figure 1). The combination of SN55 and SN62 should generate a 1.46-kb fragment that contains the RBS sequence along with the gene encoding β-lactamase.

Analysis of the products obtained by cleavage of pUC19 with SN55 and SN62 reveals that the combination of both nucleases produced greater than 60% conversion of pUC19 to two fragments (Figure 2, lane 5).[9] The larger fragment containing the β-lactamase gene was determined to be approximately 1.5 kb by comparison with molecular weight standards. It is known that these semisynthetic nucleases produce asymmetric cleavage of duplex DNA at the 5′ side of the homopurine binding site to give products with 3′ phosphate and 5′ hydroxyl termini.[3] Consequently, the 1.5-kb fragment was treated with T4 DNA kinase at pH 6.2 in the presence of ATP to remove the 3′ phosphates and phosphorylate the 5′ termini.[10] The ends were filled in by the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of nucleotide triphosphates (dNTPs).

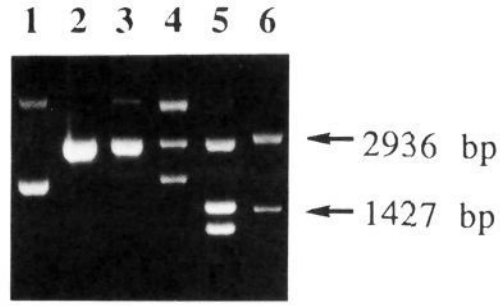

Figure 2. Cleavage of plasmid pUC19 by semisynthetic nucleases as analyzed by agarose gel electrophoresis. Lane 1, untreated pUC19 (open circular and covalently closed circular); lane 2, *Eco*RI linearized pUC19; lanes 3 and 4, circular pUC19 treated with semisynthetic nucleases SN55 and SN62, respectively; lane 5, circular pUC19 treated with both SN55 and SN62; lane 6, molecular weight standards (2936 bp and 1427 bp). Approximately 1 μg of DNA was loaded on each lane of a 1% agarose gel containing ethidium bromide.

Ligation of the 1.5-kb DNA containing the β-lactamase gene (~0.5 μg) with the dephosphorylated *Sma*I (blunt end) fragment of pDP23 (~0.5 μg) (Figure 1) places the β-lactamase gene under the control of the *lac* promoter (plasmid pAMP6, Figure 1). Transformation of competent *E. coli* TG1 cells[11] resulted in 4×10^3 colonies when plated on the kanamycin media and ~160 colonies on ampicillin/IPTG media.[12] For comparison, ligation of a 1.3-kb *Ssp*I/*Alw*NI restriction fragment of pUC19 (containing the same β-lactamase gene) into the *Sma*I site of pDP23 (under the same conditions) resulted in 5×10^3 colonies on the kanamycin media and ~1400 colonies on the ampicillin/IPTG media. A likely explanation for the 10-fold-lower cloning efficiency of the semisynthetic nucleases is that the termini were not completely blunt ended, reducing the efficiency of the subsequent ligation reaction.

Four clones, pAMP6–9, from the ampicillin/IPTG plate were further characterized by restriction mapping and double-strand dideoxy sequencing. All four clones contained the β-lactamase gene inserted at the *Sma*I site of pDP23, and the DNAs had the expected size (5.9 kb), as compared to molecular weight standards. Analysis of the DNA sequences flanking the ligation sites in these four clones revealed that SN55 and SN62 cleave plasmid pUC19 at multiple nucleotides adjacent to the 5′ termini of the homopurine binding sites. The nuclease SN55 cleaved the homopurine-containing strand of pUC19 at A 2498, T 2501, and A 2503; SN62 cleaved the homopyrimidine-containing strand at T 1039, C 1040, and G 1041 (Figure 1). The exact cleavage sites on the opposite strands could not be determined from the sequences of pAMP6–9 due to the Klenow step.

The above experiments not only demonstrate that chemical and biological mutagenesis can be used to introduce a high level of binding specificity into proteins but may also provide useful tools for the manipulation of nucleic acids.

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(12) We have also succeeded in cloning the β-lactamase gene from pUC19 into the *Eco*RI/*Alw*NI site of pSG7 and the *Ssp*I/*Ase*I site of pBR322, both of which contain the origin of replication of a relaxed plasmid pMB1.

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Supplementary Material Available: A listing of synthetic, cleavage, and ligation methods as well as the sequences flanking the ligation sites in pAMP6-9 (3 pages). Ordering information is given on any current masthead page.

Observation of the Cu_A-Ligand Stretching Resonance Raman Band for Cytochrome *c* Oxidase†

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Cytochrome *c* oxidase, the terminal enzyme of the respiratory chain of aerobic organisms, contains two heme A groups and two copper atoms as redox-active metal centers.[1] While one copper atom (Cu_B) in the resting enzyme is EPR silent as a result of antiferromagnetic coupling with one of the heme groups (Cyt a_3), the other copper atom (Cu_A) displays an atypical EPR signal in the sense that no hyperfine splittings are clearly resolved and the *g* values are quite low.[2] Although the reduced hyperfine coupling is reminiscent of type I copper (blue copper) proteins, the systematic investigation of EPR spectra of Cu-containing proteins[3] displayed an appreciable difference between Cu_A and blue copper centers. This difference was also manifested by X-ray absorption data,[4,5a,6a] which indicated that upon reduction of the protein the Cu absorption edge changed from the cupric to cuprous state for blue copper centers but not for Cu_A. The EPR and ENDOR studies on isotope-substituted yeast cytochrome *c* oxidase[7] established coordination of one cysteine (Cys) and one histidine (His), at least, to Cu_A while EXAFS studies[5b,6] suggested coordination of two N (or O) and two S (or Cl) atoms to Cu_A.

Resonance Raman (RR) spectroscopy has been used extensively to characterize the blue copper proteins.[8-11] However, excitation of Raman scattering from cytochrome *c* oxidase around 590-610 nm has brought about only heme modes[12] but no Cu_A-associated bands. Although Cu_A of the resting enzyme gives a weak and broad absorption around 830 nm,[13] observation of Raman spectra in resonance with this absorption has not been successful on account of the low sensitivity of a photomultiplier in a far-red region. Therefore, we constructed a new Raman system using a CCD (charge-coupled device) detector and applied it successfully to observe the Cu_A-ligand RR bands of cytochrome *c* oxidase.

The excitation light was obtained from a Ti-sapphire laser (SP, 3900) pumped by an Ar^+ ion laser (SP, 2045). A CCD (Astromed CCD3200) detector was attached to a single monochromator (JASCO CT-50) which employed a 750-nm blazed grating with 1200 grooves/mm. In order to circumvent the charge-trap problem,[14] the short axis of the CCD tip was turned to the direction

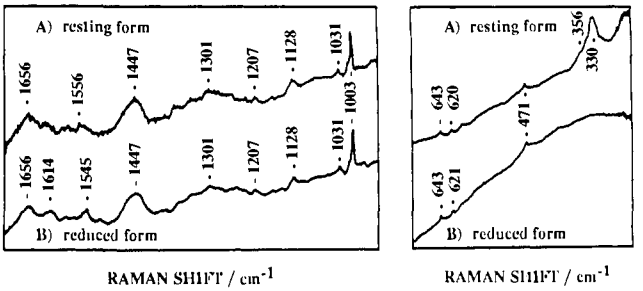

Figure 1. Resonance Raman spectra of the resting form (A) and the fully reduced form (B) of bovine cytochrome *c* oxidase excited at 840 nm. The enzyme concentration was 500 μM (in terms of Cu_A) in 10 mM sodium phosphate buffer, pH 7.4. The sample was contained in a spinning cell (1800 rpm) at about 5 °C. The reduced form was prepared by adding a small amount of dithionite anaerobically to the resting enzyme.

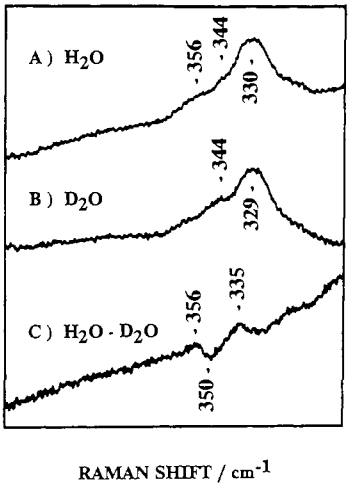

Figure 2. Resonance Raman spectra of the resting form of bovine cytochrome *c* oxidase in H_2O (A) and D_2O (B) excited at 845 nm and their difference spectrum (C = A - B). Experimental conditions are the same as those in Figure 1.

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