Nonisotopic DNA Detection System Employing Elastase and a Fluorogenic Rhodamine Substrate

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An alternative fluorescence-based method has been developed for the direct detection of small quantities of DNA in solution. In this system, a serine protease (elastase) is coupled to a DNA oligonucleotide through a disulfide linkage. A bis-(tetraalanine)-derivatized rhodamine molecule (BZTAlaR) has been synthesized for use as a substrate. BZTAlaR is nonfluorescent in its derivatized form and shows negligible hydrolysis in solution. Cleavage of the tetraalanyl groups from the rhodamine portion of the molecule restores its fluorescence. Hybridization of the elastase–oligonucleotide conjugate to its target, capture of the conjugate–target complex with streptavidin-coated magnetic beads, addition of substrate, and subsequent detection of the target by fluorescence are accomplished in solution. Hybridization is rapid and specific, with over 90% of a target sequence successfully hybridized and captured. This method exhibits low background and an amplified fluorescent signal over time, resulting in a current detection limit of 0.49 fmol of elastase alone, or 2.64 fmol of conjugate, within 2 h.

INTRODUCTION

The detection and identification of specific DNA sequences have become commonplace in recent years. DNA sequence analysis is now routinely used for the diagnosis of genetic disease, for the detection of infectious organisms, for forensic studies in criminal investigations, and in archaeological research.[1-3] Obtaining consistent and reliable results, however, is often made difficult by the fact that the amount of recoverable DNA suitable for analysis is limited. Direct detection of very small quantities of DNA requires highly sensitive detection methods. The traditional and most widely used detection procedure employs oligonucleotide probes labeled with a radioisotope such as ^{32}P or ^{35}S. Unfortunately, radioactive methods have a number of drawbacks, including long film exposure times, assay procedures that are often tedious and cumbersome, and significant health hazards. These problems have driven the development of alternative nonisotopic detection methods.[4] Many of these methods are based upon the use of an enzyme (e.g., alkaline phosphatase or horseradish peroxidase) coupled covalently to an oligonucleotide. Detection of the enzyme–oligonucleotide conjugate is accomplished by addition of a chromogenic,[4] fluorogenic,[5] or chemiluminogenic[6] substrate. The enzyme converts the substrate from a colorless, nonfluorescent, or nonluminescent form to a colored, fluorescent, or luminescent form, respectively, thereby indicating the conjugate's presence. The ability of one enzyme molecule to turn over many substrate molecules is, in effect, an amplification of the signal. This amplification gives the methods potential for great sensitivity. However, in practice these approaches are limited in their sensitivity by both the limited detectability of the products formed and a background signal level which can be high, in part due to the susceptibility of the substrates to nonenzymatic hydrolysis.[7]

In this work, an alternative nonisotopic detection system is explored which addresses some of these limitations. The work is based upon the properties of a class of protected rhodamine derivatives, in which amino acid protecting groups are attached to the two exocyclic amino groups of Rhodamine 110[8-10] (Figure 1), thereby quenching the dye's fluorescence. The protecting groups may be designed to be subtrates for a desired serine protease. The product of cleavage under conditions of substrate excess is a monoderivatized rhodamine, which is an excellent fluorophore excited with high efficiency in the visible region of the spectrum. The use of amide linkages in the fluorogenic substrate, in place of the phosphoester or ester linkages which have been most widely employed,[4-5,11] potentially increases the stability of the substrate to nonenzymatic hydrolysis, thereby reducing background levels. The serine protease employed in the present study is elastase, and the substrate a bis(tetraalanine)-derivatized rhodamine molecule (BZTALaR). The detection limit obtained in this system is 0.49 fmol for elastase alone and 2.64 fmol for the elastase–oligonucleotide conjugate.

EXPERIMENTAL SECTION

Reagents. The following materials were purchased from the indicated companies: porcine pancreatic elastase (Worthington); 2-iminothiolane hydrochloride and NHS-S-S-Biotin (Pierce); Sephadex G-25 (Pharmacia); Rhodamine-110 (Kodak); (benzyoxycarbonyl)dialanine (Cbz-Ala-Ala), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC; Sigma); 30% HBr, 30% NH_4OH (Aldrich); tetrazole, I_2/Lut/THF/H_2O, Ac_2O/Lut, and 3% dichloroacetic acid/dichloromethane solutions for the DNA synthesis reactions (Beckman); fluorescein-5-maleimide and fluorescein

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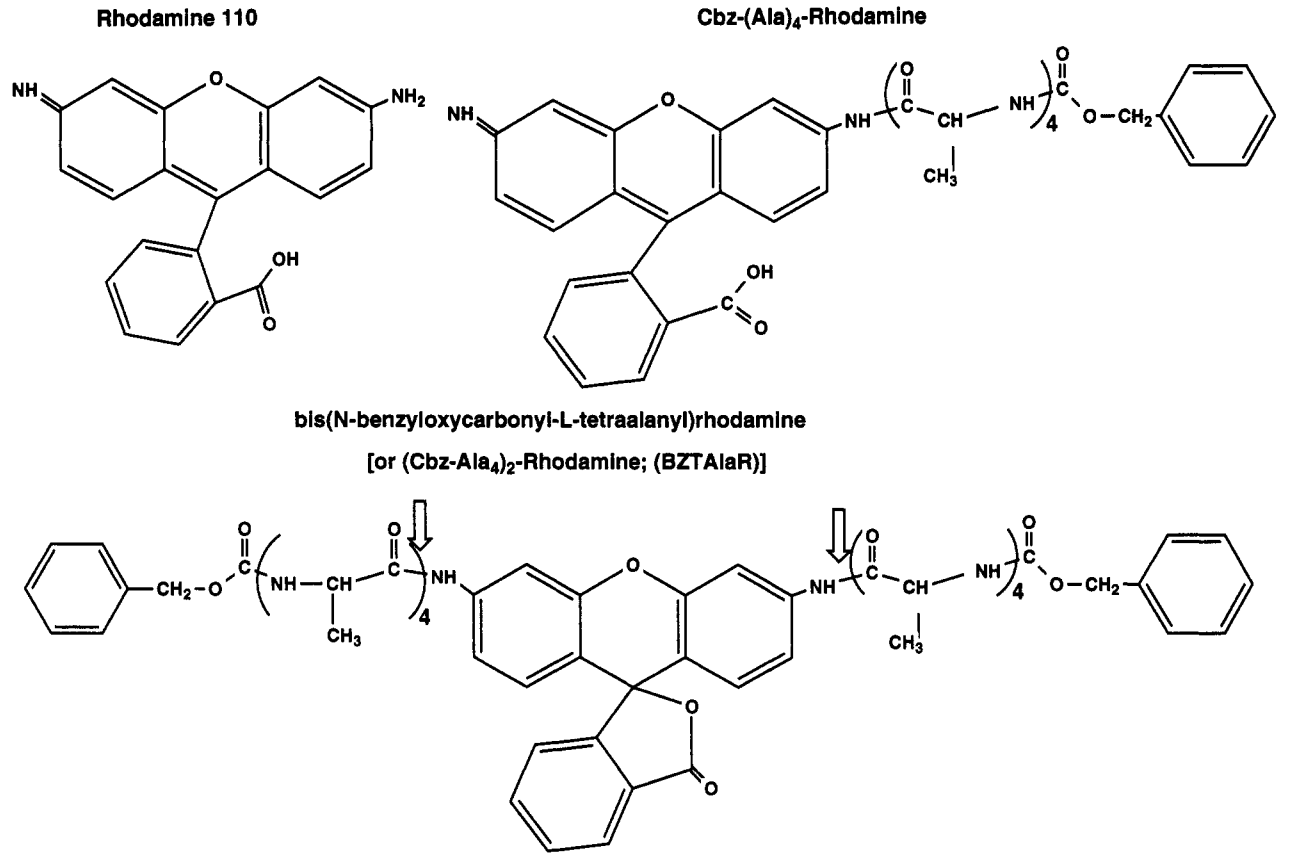

Figure 1. Molecular structures of Rhodamine 110, Cbz-(Ala)$_4$-Rhodamine (the product of enzymatic hydrolysis of BZTAlaR by elastase), and BZTAlaR. The side chains of BZTAlaR are cleaved by elastase at the positions indicated by the arrows.

isothiocyanate (Molecular Probes); streptavidin magnetic Dynabeads M-280 (Dynal A.S., Oslo, Norway); 5′-[α-^{32}P]dATP (>400 Ci/mmol) (Amersham). The Gelcode silver staining system (Pierce) was used to visualize protein and DNA bands in polyacrylamide mobility shift gels.

Two oligonucleotide sequences were used in this work: (1) U131 (5′-T*ATGGTGCACCTGACTCCTGA-3′), a 21-mer which is coupled to the enzyme and used as the probe, and (2) C131 (5′-T*TCTCCTCAGGAGTCAGGTGCACCATGGTGT-3′), a 31-mer which functions a simple target sequence for testing the efficacy of the probe. The central 20 bases of C131 are complementary to U131. Both oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center. They were both left fully protected and attached to the synthesis support. Couplings with a 5′-(monomethoxytrityl)aminothymidine phosphoramidite were performed manually as described[12,13] to yield the corresponding 5′-amino oligonucleotides. The biotin derivative of the 5′-amino C131 oligonucleotide was synthesized using NHS-S-S-Biotin as described.[14]

Methods. *Synthesis of (Cbz-Ala$_2$)$_2$-Rhodamine (BZDAlaR).* The reaction scheme is shown in Figure 2. Mangel's procedure for synthesizing amino acid-derivatized rhodamine substrates for serine proteases was used as a starting point.[8] All reactions were run anhydrously. Anhydrous pyridine (7 mL) and anhydrous DMF (7 mL) were added to 2.2 mmol of Cbz-Ala-Ala. The resulting solution was then added to 2.2 mmol of EDC. To this mixture, 0.08 mmol of Rhodamine 110 in 2 mL of pyridine/DMF was added. The reaction mixture was stirred at 4 °C for several hours and at room temperature for 2 days. During this time, the color of the reaction mixture changed from a bright fluorescent orange to a light peach. The reaction mixture was diluted with 10 mL of 0.05 M HCl and extracted four times into ethyl acetate. This phase was washed twice with saturated $NaHCO_3$, once with distilled water, and once with saturated NaCl. After washing, the solution was dried over $MgSO_4$ and reduced by rotary evaporation. Final purification was accomplished by flash chromatography, using ethyl acetate as the running solvent.[15] Column fractions were collected and analyzed by TLC under 254-nm light, using either 8:1:1 2-butanone/H_2O/acetone or ethyl acetate as the developing solvent. Fractions containing the desired product, BZDAlaR, were combined and the volume reduced by rotary evaporation to 2 mL. The product was recovered as an off-white crystalline solid by precipitation from hexane, with a yield varying between 50% and 78%. The product was judged to be pure by analytical TLC using running solvents of 8:1:1 2-butanone/H_2O/acetone (R_f = 0.75) or ethyl acetate (R_f = 0.3). BZDAlaR appears as a reddish-black spot under 254-nm UV light. There was no evidence of rhodamine (R_f = 0.4 in 8:1:1 2-butanone/H_2O/acetone and R_f = 0.028 in ethyl acetate), which appears bright green under 366-nm UV light.

Synthesis of (Ala$_2$)$_2$-Rhodamine. BZDAlaR (0.03 mmol) was added to 2 mL of 30% HBr in acetic acid in a glass centrifuge tube. The tube was sealed with a rubber septum and the reaction allowed to proceed for 40 min at room temperature under N_2, with constant stirring. The desired product, (Ala$_2$)$_2$-rhodamine, was then precipitated from diethyl ether as a fluffy, orange solid, and centrifuged at 10 000 rpm for 20 min, followed by removal of the supernatant. Resuspension of the precipitate in diethyl ether, followed by centrifugation at 10 000 rpm for 20 min, was repeated four times. The product, a hydrogen bromide salt, was judged pure by analytical TLC in 8:1:1 2-butanone/H_2O/acetone (R_f = 0.06). No starting material was evident in the product TLC. A yield of 71–84% was obtained from the blocked bis-(dialanyl) precursor.

Synthesis of (Cbz-Ala$_4$)$_2$-Rhodamine (BZTAlaR). All reactions were run anhydrously, 9 mL of anhydrous dichloromethane and 1 mL of anhydrous triethylamine were added to 0.82 mmol of Cbz-Ala-Ala. The resulting solution was combined with 0.79 mmol of EDC. This solution was added to 0.036 mmol of (Ala$_2$)$_2$-

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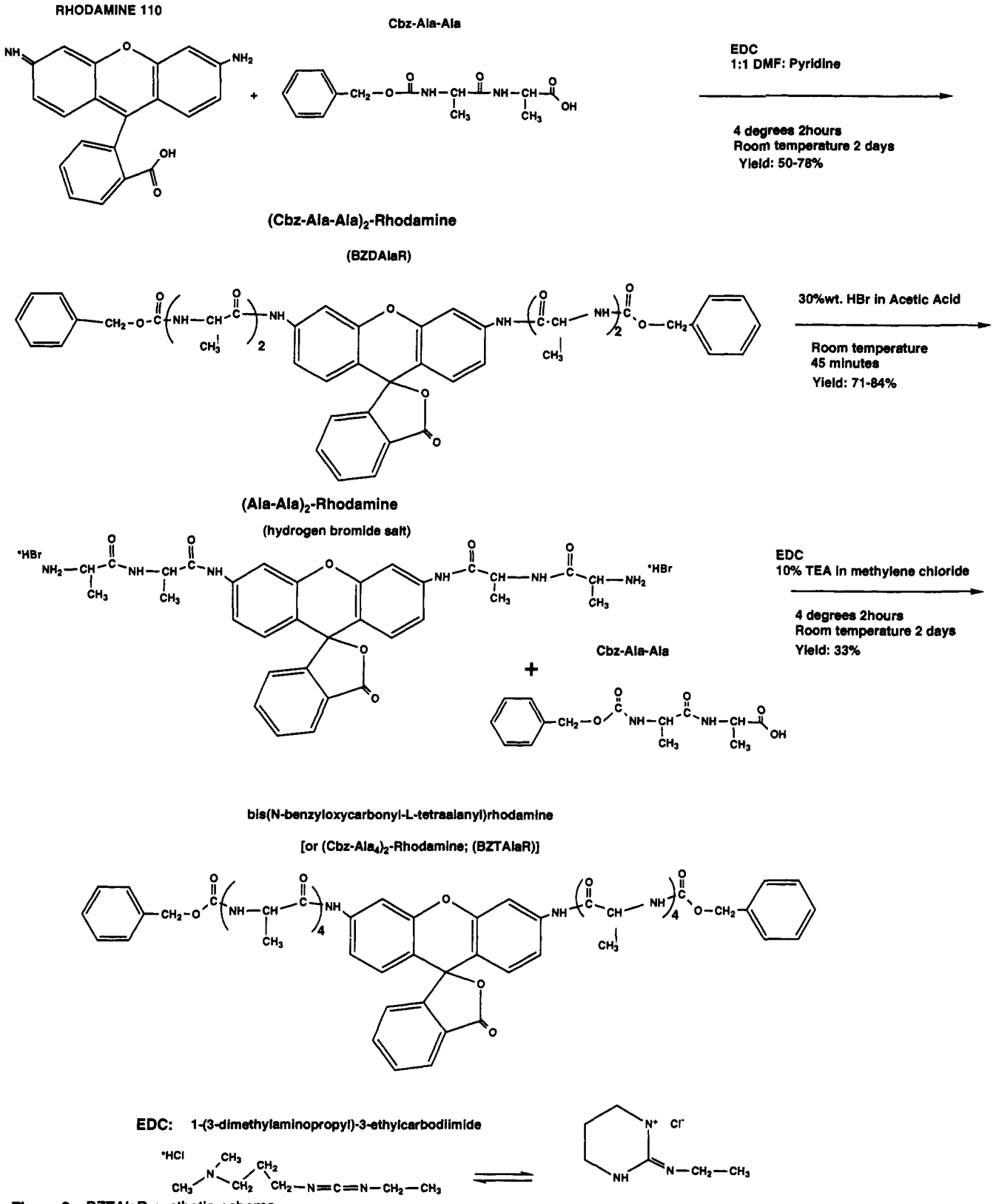

Figure 2. BZTAlaR synthetic scheme.

rhodamine. The reaction mixture was stirred at 4 °C for several hours and at room temperature for 2 days. The reaction mixture was purified by four extractions with 10 mL each of 0.05 M HCl, retaining the organic phase. This phase was then washed twice with saturated $NaHCO_3$, once with distilled water, and once with saturated NaCl. After washing, the solution was dried over $MgSO_4$, the volume reduced by rotary evaporation, and the residue dried under vacuum for several days. The desired product, BZTAlaR, was obtained as a residue, taken up in acetonitrile, and quantitated by UV absorbance at 284 nm using a molar absorptivity estimated from the spectra of known concentrations of BZAlaR in acetonitrile (ϵ_{284} = 9235 ± 1173 M^{-1} cm^{-1}). The acetonitrile was then removed by rotary evaporation, and the BZTAlaR residue was brought up to the desired volume in DMF. The product was judged pure by analytical TLC. Only one dark spot was seen under 254-nm UV light in 8:1:1 2-butanone/H_2O/acetone (R_f = 0.52), in 1:1 methanol/ethyl acetate (R_f = 0.76), or in 8:2 ethyl acetate/methanol (R_f = 0.71). No evidence of either rhodamine or the starting material, $(Ala_2)_2$-rhodamine, was present in the product TLC: ^1H NMR (200 MHz, $(CD_3)_2CO$)

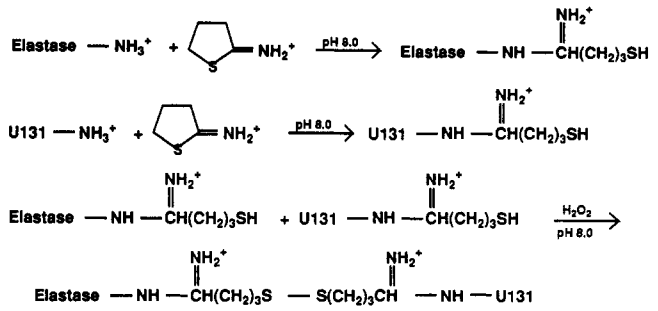

Figure 3. Elastase–oligonucleotide conjugate reaction scheme.

δ 7.35 (s, 10H, PhH), 5.1 (s, 4H, PhCH$_2$O), 1.1–1.3 (m, 24H, CH$_3$); the BZTAlaR/DMF solution was stored at −20 °C; yield, 33% from the deblocked bis(dialanyl) precursor; overall product yield from Rhodamine 110, 12–25%.

Synthesis of Cbz-Ala$_2$-Rhodamine. All reactions were run anhydrously. A 2-mL aliquot of anhydrous pyridine and 2 mL of anhydrous DMF were added to 0.20 mmol of Cbz-Ala-Ala. The resulting solution was then added to 0.30 mmol of EDC. This solution was added to 0.14 mmol of Rhodamine 110. The reaction mixture was stirred at 4 °C for several days and purified by flash chromatography[15] using 8:2 dichloromethane/methanol as the running solvent. Column fractions were collected and analyzed by TLC under 366-nm UV light, using 8:1:1 2-butanone/H$_2$O/acetone as the developing solvent. Fractions containing the desired product, Cbz-Ala$_2$-rhodamine, were combined, concentrated to dryness, dissolved in 1 mL of DMF, and precipitated from 1.0 M HCl overnight at 4 °C in a glass centrifuge tube. The tube was then centrifuged at 10 000 rpm for 20 min, followed by removal of the supernatant. The red-orange precipitate was then dissolved in 1 mL of methanol, reprecipitated from 20 mL of diethyl ether, centrifuged at 10 000 rpm for 20 min, followed by removal of the supernatant, and dried under vacuum for several days. The product was judged to be pure by analytical TLC using a developing solvent of 8:1:1 2-butanone/H$_2$O/acetone (R_f = 0.25) and appears orange-green under 366-nm UV light; yield, 16% from Rhodamine 110.

Synthesis of Elastase-NH(C=NH$_2^+$)(CH$_2$)$_3$SH. The synthetic scheme employed in coupling elastase to an oligonucleotide is shown in Figure 3. Traut's reagent was used to add a free sulfhydryl group to elastase as follows. A 19.4-mg sample of 2-iminothiolane (Traut's reagent) and 200 μL of 1M Tris, pH 8.0, were combined in an Eppendorf tube. A solution consisting of 1.5 mg of elastase in 200 μL of 50 mM Tris/50 mM KCl/1 mM MgCl$_2$, pH 8.0, was added, the resulting solution vortexed, and the reaction allowed to proceed at 4 °C for 4 h. Purification was performed on a Sephadex G-25 column (5-mL bed volume), equilibrated with 50 mM Tris/50 mM KCl/1 mM MgCl$_2$, pH 8.0. Six 750-μL fractions were collected, and the absorbance at 280 nm was measured to determine which fractions contained the elastase derivative. The presence of an active sulfhydryl group was confirmed by addition of a small amount of fluorescein-5-maleimide to the fraction containing the elastase derivative, incubation of the resulting solution at room temperature for 40 min in the dark, purification of the reaction mixture on a Sephadex G-25 column (5-mL bed volume) equilibrated with 50 mM Tris/50 mM KCl/1 mM MgCl$_2$, pH 8.0, and measurement of the absorbance of each of the six collected fractions (750 μL/fraction) at 280 (for elastase) and at 494 nm (for fluorescein); yield, 75%.

Synthesis of U131NH(CH=NH$_2^+$)(CH$_2$)$_3$SH. A similar reaction was employed to add a free sulfhydryl group to the amino oligonucleotide U131-NH$_2$. A 5.3-mg sample of 2-iminothiolane, 50 μL of 1 M Tris, pH 8.0, 47 μL of 50 mM Tris, 50 mM KCl, 1 mM MgCl$_2$, pH 8.0, and 3 μL of 2.5 nmol/μL U131-NH$_2$ (total 7.5 nmol) were combined in an Eppendorf tube, vortexed, and allowed to react at 90 °C for 4 h. The reaction mix was purified in the same manner as the elastase derivative. The absorbance of each of the six collected fractions (750 μL/fraction) at 260 nm was measured to determine which fractions contained the derivatized oligonucleotide. The presence of an active sulfhydryl group was confirmed in the same manner as for the elastase derivative, using the absorbance at 260 nm instead of 280 nm to determine presence of oligonucleotide; yield, 67–83%.

Synthesis of Elastase-NH(C=NH$_2^+$)(CH$_2$)$_3$SS(CH$_2$)$_3$-(C=NH$_2^+$)NH-U131. A disulfide linkage was formed between the sulfhydryl-conjugated elastase and oligonucleotide as follows:[16] the fractions containing the elastase derivative and the oligonucleotide derivative were combined, 20 μL of 30% hydrogen peroxide was added to initiate the oxidation of the sulfhydryl groups, and the sample was vortexed and incubated at 4 °C for 2–2.5 h. Purification of the reaction mixture was performed on a Pharmacia FPLC MonoQ anion exchange column, equilibrated with 100 mM piperazine, pH 5.0, for 38 min with a 0–1 M NaCl gradient at a flow rate of 1.0 mL/min. The elastase–oligonucleotide conjugate fractions were concentrated to about 400 μL by centrifugation using an Amicon Centricon-10 (10 000 MW cutoff) filter. Glycerol was added to make the final volume of elastase–oligonucleotide conjugate about 20% in glycerol. In some cases, conjugation between elastase and U131 was confirmed by the following two methods. First, elastase–oligonucleotide conjugate was compared with both U131-NH$_2$ and elastase on a 14% polyacrylamide native gel (pH 10.0). Protein and DNA bands in the gel were visualized using the Gelcode silver staining system (Pierce) according to the manufacturer's instructions. In these experiments, a significant difference in mobility is observed between elastase, U131-NH$_2$, and the elastase–oligonucleotide conjugate. Second, to verify that the enzymatic activity of the elastase–oligonucleotide conjugate toward BZTAlaR is due to elastase being covalently linked to U131-NH$_2$, rather than due merely to nonspecific binding of elastase to U131-NH$_2$, the following experiment was performed. Unmodified elastase and U131-NH$_2$ were combined under the usual conjugation reaction conditions. The U131-NH$_2$ was then purified in the same manner as that performed for the elastase–oligonucleotide conjugate. The resulting sample was analyzed for elastase activity in a microtiter plate using BZTAlaR, the fluorescence being monitored at room temperature using a Pandex fluorescence concentration analyzer, with excitation at 485 nm and emission at 525 nm. The U131-NH$_2$ was observed to have negligible enzymatic activity toward BZTAlaR, thereby verifying that elastase and U131 are covalently linked together in the conjugate. The ratio of elastase to U131 in the conjugate was determined by comparing the UV absorbance of the conjugate at 260 and 280 nm. The results obtained were consistent with that expected for a 1:1 coupling ratio; yield, 24%.

Determination of the Rate of Nonenzymatic Hydrolysis of BZTAlaR. The rate of nonenzymatic hydrolysis for BZTAlaR in the fluorescence kinetics assay buffer was determined as follows. A solution of 450 μM BZTAlaR in 10 mM Tris/20 mM CaCl$_2$/18% DMF, pH 8.8 was prepared and the fluorescence increase monitored at 25.0 °C over 2 h using an SLM 8000C spectrofluorometer, with excitation at 494 nm and emission at 530 nm. The hydrolysis rate was determined by averaging the results of three trials.

Determination of Kinetic Parameters of BZTAlaR. The kinetic parameters, k_{cat} and K_M, for BZTAlaR cleavage by elastase or elastase–U131 conjugate was determined as follows. Four assay solutions, one each of concentration 60, 150, 300 and 450 μM BZTAlaR in 10 mM Tris/20 mM CaCl$_2$, pH 8.8/18% DMF, were prepared for each trial. A kinetics assay was run by addition of an aliquot of either elastase or elastase–U131 conjugate in 10 mM piperazine, pH 5.0, to an assay solution to make it either 0.013 μM active elastase or 0.0036 μM active elastase–U131 conjugate. The fluorescence of the solution was monitored at 25.0 °C over 5 min using an SLM 8000C spectrofluorometer, with excitation at 494 nm and emission at 530 nm. The BZTAlaR concentration range used was between about 0.2K_M and 2K_M.[17] The elastase/elastase–U131 conjugate concentration was chosen to ensure that, under these conditions, the increase in measured fluorescence with time remained linear, and so that no more than 5% of the substrate was converted to monosubstituted product by elastase.[9] The measured fluorescence was related to the concentration of monosubstituted product by using a standard curve produced from the measured fluorescence of a set of serial dilutions of Cbz-Ala$_2$-rhodamine in 10 mM Tris/20 mM CaCl$_2$, pH 8.8/18% DMF, monitored at 25.0 °C in the SLM 8000C

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spectrofluorometer, with excitation at 494 nm and emission at 530 nm. The concentration of active elastase and active elastase–U131 conjugate was determined by the fluorescence titration method of Melhado et al. using fluorescein mono-*p*-guanidinobenzoate.[18] Average velocities were determined from five (elastase) or six trials (elastase–U131 conjugate), and the kinetic parameters were determined from a Lineweaver–Burk double-reciprocal plot of the data (see Results and Discussion).

Determination of the Effect of DMF on Elastase Activity. Experiments were performed to evaluate two possible deleterious effects of the DMF: (1) that the DMF was denaturing the elastase over time; (2) that the DMF, while not denaturing the elastase, was in some way reducing its turnover rate. To test the first possibility, a 1 mg/mL solution of elastase in 10 mM Tris/20 mM $CaCl_2$, pH 8.8, was prepared and divided into two equal portions. DMF was added to one solution to make it 18% in DMF. The other was used as a control. Both solutions were allowed to incubate at room temperature, and 5-μL aliquots were removed after 1, 2, and 4 h from both solutions and analyzed for elastase activity at room temperature using a *p*-nitroanilide UV absorbance assay.[19] No appreciable difference in activity was observed between the two solutions.

To test the second possibility, a 1 mg/mL solution of elastase in 100 mM Tris, pH 8.0 buffer was prepared. Elastase activity was monitored by analyzing 5-μL aliquots of this solution at room temperature in a *p*-nitroanilide UV absorbance assay containing either 18% (v/v) DMF or no DMF. The turnover rate measured in the DMF-containing assay was about one-sixth of that found in the control assay.

Determination of Hybridization Efficiency of the Probe. To ascertain the hybridization efficiency of the elastase–U131 conjugate to its target sequence in both the presence and absence of contaminating DNA, a simple hybridization assay was employed,[4] as shown in Figure 4. A 0.2-pmol sample of biotin–C131 and 0.50 pmol of elastase–U131 conjugate, labeled with ^{32}P at the 3′-end of U131, were combined in 6× SSPE/0.1% Tween 20, pH 7.4 buffer, in both the presence and absence of added salmon sperm DNA. One control containing no biotin–C131 (to test nonspecific binding of probe to beads) was also prepared. Hybridization was performed at 37 °C for times of 60, 30, 15, 7.5, and 3 min. The conjugate–target complex was then captured by addition of streptavidin-coated magnetic geads (Dynabeads), which were prewashed three times with 6× SSPE/0.1% Tween 20, pH 7.4 buffer, to the hybridization solution, followed by gentle shaking for 30 min at room temperature. The beads were immobilized to the side of the Eppendorf tube with a magnet, washed three times with 2× SSPE/0.1% Tween 20, pH 7.4 buffer, taken up in a few microliters of distilled water, and transferred to scintillation vials. Biosafe II scintillation cocktail (1 mL) was added to each vial, and the vials were counted on a Beckman scintillation counter. Hybridization efficiency for each hybridization time was determined by averaging the results of six trials for hybridization in the presence and absence of added salmon sperm DNA.

Determination of Detection Limits for Elastase and Elastase–U131 Conjugate Acting on BZTAlaR. Detection limits for both elastase and elastase–U131 conjugate were determined as follows. All assay solutions were 450 μM BZTAlaR in 10 mM Tris/20 mM $CaCl_2$/18% DMF, pH 8.8, to which serial dilutions of either elastase or elastase–U131 conjugate in 10 mM piperazine, pH 5.0, were added. The fluorescence increase was monitored at 25.0 °C over a period of 2 h in an SLM 8000C spectrofluorometer with excitation at 494 nm and emission at 530 nm. Detection limits are defined as the amount of elastase or elastase–U131 conjugate giving a fluorescence signal with a signal-to-noise ratio of 2. Detection limits were determined from the results of three trials, using two different methods as discussed under Results and Discussion.

Determination of the Detection Limit for Elastase–U131 Conjugate–C131 Target Complex Acting on BZTAlaR in a Hybridization Assay. The detection limit for elastase–U131 conjugate–C131 target complex in a hybridization assay was determined as follows. Assay solutions composed of 1:10 serial dilutions of biotin–C131 (from 500 fmol to 5 amol per solution), plus one control containing no biotin–C131 (to test nonspecific binding of conjugate to beads) in 6× SSPE/0.1% Tween 20, pH 7.4 were prepared. A 2.5-pmol aliquot of elastase–U131 conjugate was added to each solution, and the solutions were incubated for 30 min at 37 °C. The conjugate–target complex was captured by addition of streptavidin-coated magnetic beads (Dynabeads) to the hybridization solutions, followed by gentle shaking for 30 min at room temperature. The beads were then immobilized with a magnet to the side of the Eppendorf tube, washed three times with 2× SSPE, pH 7.4 buffer, followed by addition of 30 μL of BZTAlaR solution (450 μM BZTAlaR in 10 mM Tris/20 mM $CaCl_2$/18% DMF, pH 8.8 buffer) to the beads. The resulting BZTAlaR/bead mixtures were transferred to a microtiter plate, and the fluorescence increase was monitored at room temperature over time with a Pandex fluorescence concentration analyzer, with excitation at 485 nm and emission at 525 nm.

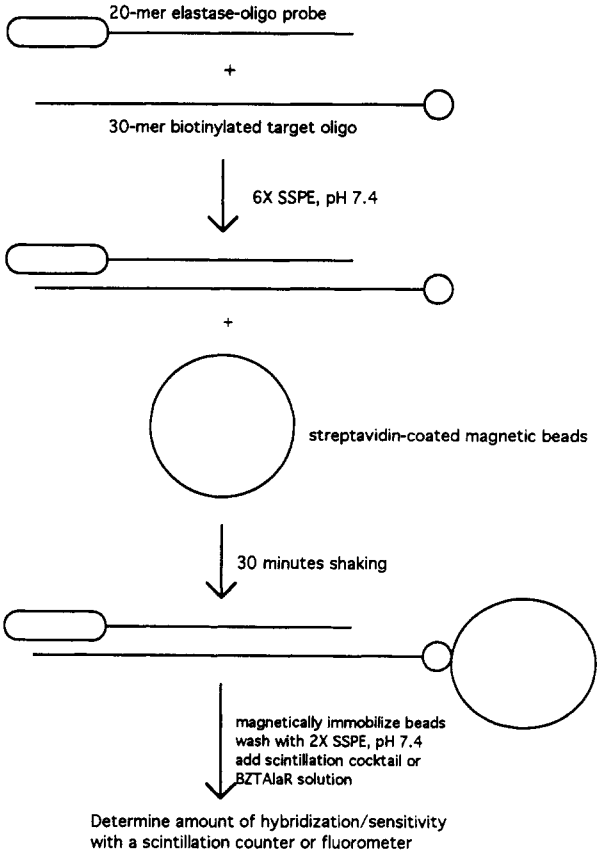

Figure 4. Schematic of "mock" hybridization assay used to determine hybridization efficiency and the sensitivity of the elastase–oligonucleotide conjugate for its target.

RESULTS AND DISCUSSION

General Strategy. Many nonradioactive methods for detecting DNA in hybridization assays are based upon the enzymatic cleavage of a substrate containing a phosphoester linkage, e.g., *p*-nitrophenyl phosphate or 4-methylumbelliferyl phosphate.[4,20] One major problem with these substrates is the susceptibility of phosphoester linkages to significant nonenzymatic hydrolysis,[7] resulting in high levels of background and a concomitant loss in sensitivity. One approach to this problem is to design the substrates in such a way as to maximize the hydrolytic stability of the phosphoester linkage. This approach was used successfully in the development of improved chemiluminogenic substrates for alkaline

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phosphatase, such as the compound 3-(2′-spiroadamantane)-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD).[6]

An alternative approach is to utilize a different enzyme–substrate combination, in which the substrate is intrinsically more resistant to hydrolysis. Such an approach was described by Mangel et al. in a series of papers describing the properties of amino acid-derivatized rhodamines as fluorogenic substrates for a variety of serine proteases. Mangel et al. demonstrated that one could "tune" the rhodamine substrates specifically for a desired protease by varying the nature of the amino acid protecting groups.

Choice of Serine Protease. The attractive properties of these fluorogenic substrates suggested that they might be good candidates for use in nonisotopic DNA diagnostic assays. Coupling of an appropriate serine protease to an oligonucleotide would permit use of the resulting conjugate in a variety of assay formats. A critical element in this approach is the choice of serine protease. An ideal enzyme for this application would be very stable to autolysis and have a high turnover number and a low K_M for the substrate. Initial work with trypsin was unsuccessful due to autolytic degradation. Both elastase[21] and α-lytic protease[22] were suggested (see Acknowledgment) as being substantially more stable. Literature reports suggested that, although α-lytic protease was extremely stable to autolysis, it also had a very low turnover number, which would compromise its sensitivity in a DNA diagnostic assay.[23] Elastase, however, appeared to have both excellent stability and a reasonably high turnover number and was thus chosen for further work.[24,25]

Elastase preferentially cleaves peptides at amino acids containing small aliphatic side chains, especially alanine, in the P_1 position, the position preceding the scissile bond.[11] As for most serine proteases, the catalytic activity of elastase can be controlled by solution pH.[21] This is important because, while elastase exhibits little or no activity at pH 5.0, at pH values near its catalytic maximum (pH 8.8) significant autolysis and loss of enzymatic activity are observed. Controlling the catalytic activity of elastase by working at a low pH during purification facilitates the synthesis of a stable enzyme–oligonucleotide conjugate. The chemistry employed for coupling of the enzyme to an oligonucleotide is diagrammed in Figure 3 and described in the Experimental Section.

Substrate Design and Synthesis. Kasafirek et al. reported that both the specificity and the rate of elastase activity increase with additional alanine residues (in the P_2, P_3, and P_4 positions[26]) in substituted *p*-nitroanilides, which can serve as synthetic absorbance substrates for elastase.[24] Consequently, a bis(tetraalanyl) derivative of rhodamine was chosen to "tune" the rhodamine substrate specifically for elastase. Synthesis of this fluorogenic elastase substrate, abbreviated BZTAlaR, was performed in three steps, as described in the Experimental Section.

Kinetic Characterization. The kinetic parameters of the enzyme–substrate combination are a critical element in this or any other enzymatic amplification method. The kinetic behavior of elastase (or any trypsin-like serine protease) is described by the Michaelis–Menten model[27] and results in the following rate equation:

$$v = (V_{max}[S])/([S] + K_M) \quad (1)$$

where v is the rate of substrate turnover, $V_{max} = k_{cat}[E_T]$, k_{cat} is the catalytic rate constant or turnover number, which equals the number of substrate molecules converted into product per unit time when the enzyme is saturated with substrate, $[E_T]$ is the total active enzyme concentration, [S] is the substrate concentration, and K_M is the Michaelis constant, which equals the substrate concentration at which v is 50% of maximum. The above equation is often rewritten as the following linear equation:

$$1/v = (1/V_{max}) + (K_M/V_{max})(1/[S]) \quad (2)$$

A plot of the reciprocal of the reaction velocity as a function of the reciprocal of the substrate concentration, known as a Lineweaver–Burk double-reciprocal plot,[28] permits determination of the value of K_M and V_{max}, and thereby the value of k_{cat} as well.

A high turnover number k_{cat} is necessary for generation of a large fluorescence signal in a short time. A low K_M permits a minimum amount of substrate to be employed, reducing cost as well as decreasing fluorescence background. A low K_M is also important in the present work, as the BZTAlaR substrate is poorly soluble in pure water. These kinetic parameters were determined for both elastase alone and the elastase–oligonucleotide conjugate. Data collected from the kinetics assay experiments described above were analyzed using Lineweaver–Burk double-reciprocal plots (Figure 5a,b). The kinetic parameters calculated from these data are shown in Table I.

A comparison of the k_{cat} values determined for elastase alone or for the elastase–oligonucleotide conjugate (Table I) shows that the catalytic activity of active elastase conjugate is about one-third of that for active elastase alone. This result is not surprising; comparable reductions in the specific activity of the enzyme after conjugation with an oligonucleotide commonly occur[16] and likely result from distortions in the normal tertiary structure (and therefore the active site) of the enzyme caused by conjugation of the enzyme to the oligonucleotide. Nevertheless, the reasonably high k_{cat} obtained for the conjugate indicates a good fit of the substrate in the enzymatic active site and a good turnover of the substrate.

As discussed above, the nonenzymatic hydrolysis rate of the substrate is a critical parameter of the system. This rate was measured as described in the Experimental Section, and a value of $v_{hyd} = 5.76 \times 10^{-4} \pm 0.03 \times 10^{-4}$ μM/s was obtained. The half-life of this substrate is therefore 109 h at 25 °C, which is comparable to the published value for the half-life of 110 h at 30 °C for AMPPD.[6]

Substrate Solubility. As in any enzymatic reaction, the concentration of substrate can affect the reaction rate. At a substrate concentration equal to the K_M, the reaction proceeds at $^1/_2$ the maximum velocity V_{max}. Lower substrate concentrations mean lower reaction rates and decreased sensitivity. For this reason it is desirable to use a substrate concentration substantially above the K_M in the assay itself. The substrate BZTAlaR, however, is very hydrophobic due to the eight alanyl residues; consequently, BZTAlaR was found to be poorly soluble in aqueous solution. A number of water-miscible organic solvents were therefore examined for their ability to increase BZTAlaR solubility without adversely affecting the elastase. The inclusion of 18% by volume of *N*,*N*′-dimethylformamide (DMF) in the assay solutions was found to increase solubility of the BZTAlaR substrate to about 840 μM, well above the substrate K_M.

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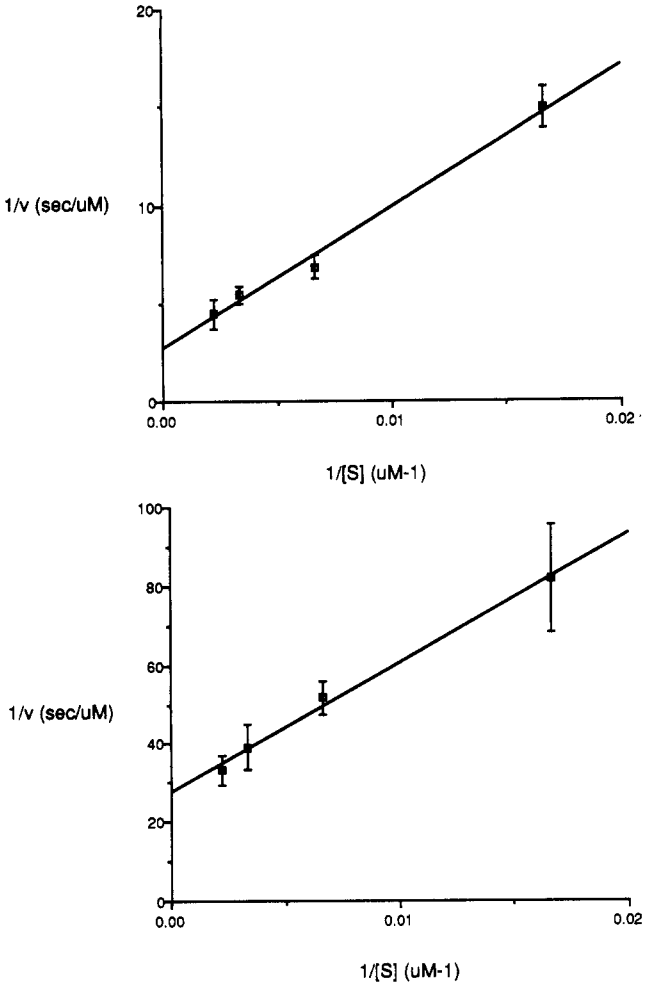

Figure 5. Lineweaver–Burk double-reciprocal plots of the kinetics data obtained for the (a, top) elastase–BZTAlaR and (b, bottom) elastase–oligonucleotide conjugate–BZTAlaR systems. Kinetic parameters for each system were determined from their respective regression lines: (a) $y = (2.67 \pm 0.67\ s/\mu M) + (722.74 \pm 48.79\ s)x$, $R^2 = 0.991$; (b) $y = (27.72 \pm 4.58\ s/\mu M) + (3288.0 \pm 171.1\ s)x$, $R^2 = 0.995$.

Table I. Kinetic Parameters

	elastase–BZTAlaR	conjugate–BZTAlaR
V_{max} ($\mu M\ s^{-1}$)	0.37 ± 0.09	0.04 ± 0.01
K_M/V_{max} (s)	722.7 ± 48.8	3288.0 ± 171.7
k_{cat} (s^{-1})	29.2 ± 7.3	10.0 ± 2.7
K_M (μM)	268 ± 69	119 ± 21

Experiments performed to evaluate two possible deleterious effects of the DMF on elastase showed that, while DMF does not appear to denature elastase per se, it does reduce the turnover rate by a factor of 6 (see Experimental Section). The latter result suggests that a fruitful area for further work would be the synthesis of an alternative substrate containing one or more hydrophilic amino acids at the ends of the tetraalanyl side chains to increase the substrate's solubility in aqueous solution, thereby eliminating the need for an added organic modifier and increasing the specific activity of the elastase. The elimination of the necessity for added DMF would presumably lead to about a 6-fold increase in the value of k_{cat}, with a concomitant decrease in the measured detection limits (see below for discussion).

Hybridization Assay. A variety of assay formats have been developed for the detection of genetic mutations (reviewed in ref 1), most of which are compatible with the elastase–BZTAlaR detection system described above. In the present work we chose to test the detection system using the simple mock assay diagrammed in Figure 4.

Table II. Detection Sensitivity[a]

system	SLM8000C 2 h	Pandex FCA 4 h	Pandex FCA 14 h
elastase	1.54 (EP) 0.49 (S)	1	0.35
elastase-U131 conjugate	7.11 (EP) 2.64 (S)	10	5
conjugate/target complex		500	50

[a] Detection sensitivities were determined by either the end point (EP) or slope (S) method at a signal-to-noise ratio of 2. All values are given in femtomoles.

Efficiency and specificity of hybridization are two key issues that must be addressed in designing a reliable assay system. It is imperative that the enzyme–oligonucleotide conjugate hybridize to its target in a reasonably short period of time, if both the overall analysis time and the degradation of the enzyme portion of the conjugate are to be minimized. In addition, the hybridization of conjugate to target must be highly specific for the particular DNA sequence being analyzed, to minimize "false positives".

Our elastase–oligonucleotide conjugate satisfies both of the above criteria. To test hybridization efficiency, a series of hybridization/capture experiments were performed (see Experimental Section). An efficiency of about 90% was obtained in all cases. Nonspecific binding of elastase–oligonucleotide conjugate to the streptavidin-coated magnetic beads was negligible. The insignificant effect of hybridization time on binding efficiency is presumed to reflect (a) the fact that hybridization is also occurring during the secondary 30-min capture period and (b) the relatively rapid rate of hybridization in free solution, particularly compared to hybridization on a nitrocellulose filter.[29]

Hybridization specificity was tested by repeating the above experiments, but this time "contaminating" the assay with nonspecific salmon sperm DNA. We observed no significant difference in hybridization rate between this case and the "clean" case (data not shown). This indicates that our elastase–oligonucleotide conjugate hybridizes to its proper target with high specificity.

Detection Sensitivity. One of the most important practical aspects of this or any other nonisotopic detection system is detection sensitivity. Sensitivity may be quantified by determining the amount of target which results in a signal-to-noise ratio of 2, the "detection limit". While the sensitivity of this system depends upon several variables, most fundamentally, it is the relative rates of enzymatic and nonenzymatic hydrolysis of the substrate that determine how much of the fluorescence signal produced as a function of time is due to the presence of the oligonucleotide-conjugated elastase (and hence target nucleic acid) and how much is nonspecific background.

Detection limits were determined after both a 2-h and an overnite (14-h) incubation, using two different fluorescence instruments, the very sensitive photo-counting SLM8000C spectrofluorometer and a relatively simple and inexpensive fluorescence microtiter plate reader, the Pandex FCA. They were determined for elastase alone, elastase-conjugated oligonucleotide alone, and for elastase-conjugated oligonucleotide employed in the mock direct hybridization assay described above. The results obtained are presented in Table II. A comparison of these values with published values for the detection limits of alkaline phosphatase and horseradish

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peroxidase-conjugated DNA oligonucleotides shows that the elastase–BZTAlaR system is comparable to the alkaline phosphatase–*p*-nitrophenyl phosphatase system, and about 1 order of magnitude less sensitive than the horseradish peroxidase–*o*-phenylenediamine system.[4] Further improvements in the sensitivity of the elastase–BZTAlaR system necessitate increasing the turnover number of elastase for the substrate. This can be accomplished by redesigning the substrate to eliminate the need for DMF in the assay (as discussed previously) or by engineering elastase to increase the specificity of its active site for the substrate. Redesign of enzyme structure may be accomplished using site-directed mutagenesis techniques.[30]

Two different methods were employed in the determination of the 2-h detection limits for elastase and elastase–oligonucleotide conjugate. In the "end point" method, the background signal after 2 h is subtracted from the signal due to both the background and the enzymatic turnover of substrate after 2 h to obtain the signal due solely to enzymatic turnover of substrate. The noise is determined by experimentally monitoring the variation in fluorescence of Rhodamine 110 with time in the SLM8000C. In the "slope" method, both the signal and the noise are obtained by fitting the fluorescence vs time data to a line by linear regression.[31]

In comparing the results obtained for the two methods (Table II), one sees that the slope method produces about a 3-fold lower detection limit than the end point method. This improvement occurs because the slope method averages the noise in the spectrum over 2 h, thereby reducing it, resulting in a concomitant improvement in both the S/N ratio and the limits of detection.

CONCLUSIONS

A novel, fluorescence-based enzymatic application system has been developed for DNA diagnostics. In this system, the enzyme elastase is coupled to an oligonucleotide and the resulting conjugate employed in a DNA probe assay. An elastase-specific fluorogenic rhodamine substrate is added to product an amplified fluorescence signal. The current detection limits are comparable to the absorbance-based alkaline phosphatase–*p*-nitrophenyl phosphate system. This work suggests straightforward improvements in substrate design would be likely to decrease detection limits by an additional factor of 6. Furthermore, an additional decrease in the detection limit may be obtainable by altering the structure of elastase to improve its specificity for the derivatized rhodamine substrate.

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