# A Fluorescent Radioiodinated Oligonucleotidic Photoaffinity Probe for Protein Labeling: Synthesis and Photolabeling of Thrombin

Catherine Berens,[†,‡] Pierre J. Courtoy,[‡] and Etienne Sonveaux*[,†]

Laboratoire de Chimie Thérapeutique et de Radiopharmacie, UCL 7340, 73 Avenue Emmanuel, Mounier, B-1200 Bruxelles, Belgium, and Unité de Biologie Cellulaire, UCL 7541, Christian de Duve Institute of Cellular Pathology, 75 Avenue Hippocrate, B-1200 Bruxelles, Belgium. Received June 10, 1998; Revised Manuscript Received October 1, 1998

To study the interactions between oligonucleotides and proteins, an original photoaffinity radiolabeling probe has been synthesized. Starting with a 5′-pyridyldithio-3′-amino-oligonucleotide, the photophore benzophenone was first coupled to the 3′ end, through acylation by an activated ester of benzoylbenzoic acid. A fluorescein molecule was grafted by alkylation of the free 5′-SH. This compound was finally radiolabeled with $^{125}$I using IodoBeads. The selective photolabeling of thrombin in a complex protein mixture by the radioiodinated probe validates this strategy to identify oligonucleotide-binding proteins.

## INTRODUCTION

The potential of oligodeoxyribonucleotides (ODNs) as antigene or antisense agents to inhibit protein expression, or as direct aptamers to proteins, is investigated increasingly. The best known examples of protein-binding ODNs are phosphodiester (PO) ODNs, featuring a G quartet, that inhibit the serine protease thrombin, a key blood-coagulation enzyme and, accordingly, a major target for anticoagulant therapy (Bock et al., 1992, Wang et al., 1993, Padmanabhan et al., 1993, Macaya et al., 1995, Shaw et al., 1995, Tsiang et al., 1995). Depending on their length and secondary structure (Tasset et al., 1997), G-quartet ODNs can bind either to the fibrinogen recognition or to the heparin-binding exosite of thrombin. Phosphorothioates (PS) ODNs, selected for their metabolic stability, avidly bind to various proteins such as recombinant soluble CD4 receptor, HIV-1 envelope glycoprotein gp120, HIV-1 reverse transcriptase, basic fibroblast growth factor (bFGF), acidic FGF, FGF-4, platelet-derived growth factor, vascular endothelial growth factor, laminin [reviewed by Stein (1995)] and more trivially, albumin (Greig et al., 1995). The sequence requirement for affinity is far less stringent for phosphorothioates than for phosphodiesters. In other words, PO−ODNs appear as unstable, but selective agents while PS−ODNs are more stable but less selective. 3′-Modified phosphodiester ODNs, resistant to 3′-exonuclease (the major serum nucleasic activity), probably feature a better compromise between stability and selectivity (Tidd and Warenius, 1989; Shaw et al., 1991).

The study of protein−ODN interactions was however not the initial focus in nucleic acid drug research. The major goal of ODNs is the antisense effect, i.e., the specific inhibition of translation of a (pre)mRNA, induced by a sequence-selective hybridization by Watson−Crick pairing with the antisense ODN. Nevertheless, whatever the nature of ODN backbone (be it PO, PS, or 3′-modified PO), an absolute requirement for an antisense effect is the cellular penetration of the ODN that presumably depends on receptor-mediated endocytosis, a process for which protein−ODN interactions are major determinants (Loke et al., 1989; Yakubov et al., 1989; Beltinger et al., 1995).

As protein−ODN interactions are so ubiquitous, we aimed at devising a standard procedure to synthesize ODN-affinity probes that would allow for specific and efficient labeling of target proteins. A photo-cross-linkable probe was favored because it could enable one to study in situ time-dependent phenomena such as the cellular penetration of ODNs. This tool would in particular prove valuable to characterize the series of protein associations of an antisense ODN on its journey from the cellular surface to the endosomes (early or late), and from the cytosol to the nucleus [reviewed by Akhtar and Juliano (1992)].

This report describes the synthesis, characterization, and radioiodination of an ODN coupled to the photophore benzophenone. This probe was further evaluated in vitro by photolabeling human and bovine thrombin. In cell biological experiments, this fluorescent ODN appears suitable for time-resolved (by flashing at different times) and space-resolved (by using confocal microscopy) chemoselective studies (by affinity labeling).

## EXPERIMENTAL PROCEDURES

**General.** Unless otherwise stated, all reagents used for this study were of analytical grade. All buffers were made with milliQ water (Millipore). The 5′-pyridyldithio-3′-amino-oligonucleotide (ODN1, see Figure 1) was obtained by the method of Arar et al. (1995). The disulfide phosphoramidite building block and the suitably derivatized glass beads required for 5′ and 3′ modifications respectively are available from Glen Research (VA, Thiol Modifier C6 S−S and 3′-Amino-Modifier C7 CPG). High-performance liquid chromatography was performed on a Spectra Physics apparatus, using reversed-phase C18 columns (BioSil C18 HL 90-5S from Bio-Rad, or Adsorbosphere from AllTech) at a flow rate of 1 mL/min and a linear gradient of eluent B (triethylammonium acetate (TEAA) 0.1 M pH 7.0 in $CH_3CN$:$H_2O$, 60/40) in eluent A

* To whom correspondence should be addressed. Phone: 32.2.764.54.61. Fax: 32.2.764.73.63. E-mail: sonveaux@cmfa.ucl.ac.be.

† Laboratoire de Chimie Thérapeutique et de Radiopharmacie.

‡ Unité de Biologie Cellulaire.

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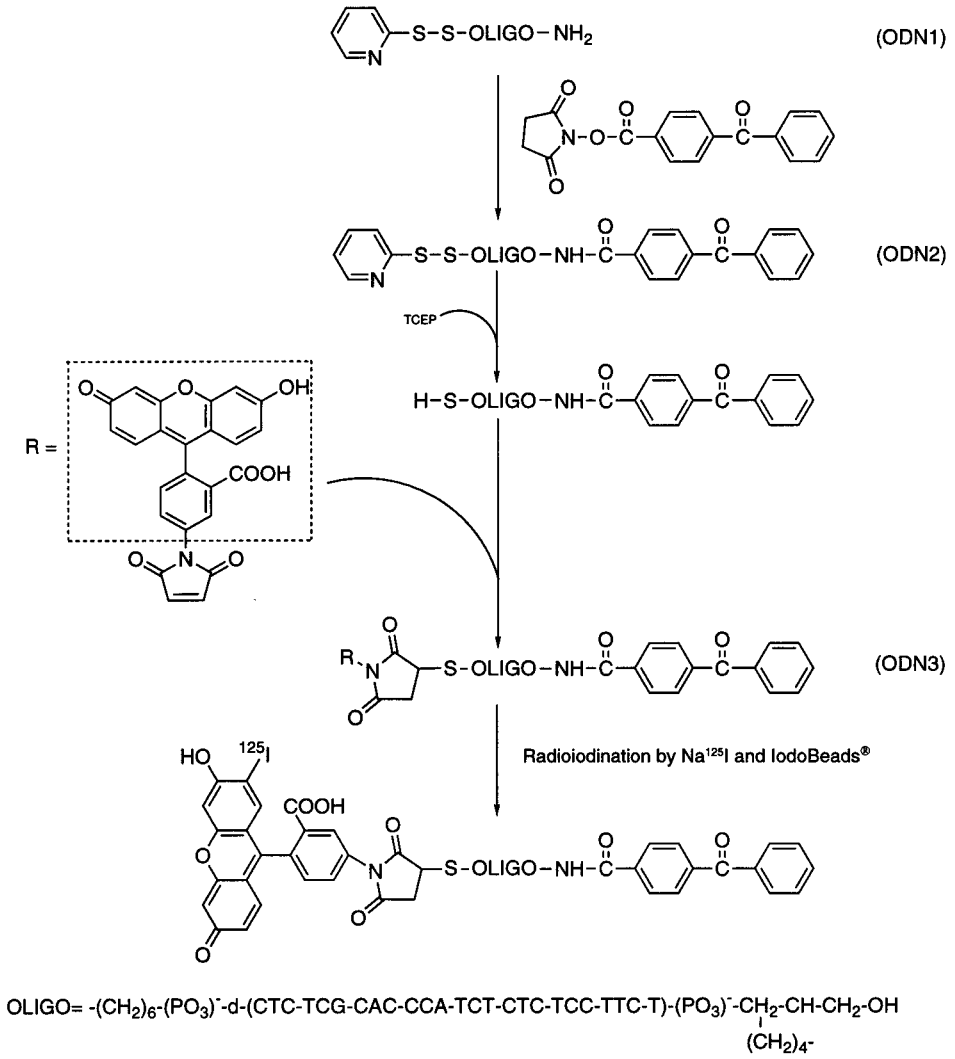
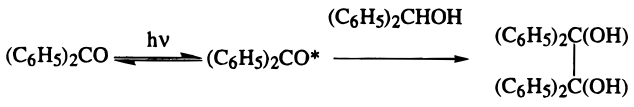



**Figure 1.** Structures and synthesis of ODNs. [TCEP is tris(2-carboxyethyl)phosphine].

(0.1 M TEAA, pH 7.0, in $H_2O$:$CH_3CN$, 95/5) from 0 to 100% over 60 min. Aqueous solutions were evaporated without heating on a Speed Vac centrifugal evaporator (Savant). The concentrations of purified ODNs were determined by UV spectroscopy at the maximum absorbance of the ODN (269 nm), using extinction coefficients calculated as described by Brown and Brown (1991).

For photo-cross-linking experiments, samples were exposed in open Petri dishes to illumination with three lamps (total power of 60 W, 30 W from above, 2 × 15 W lateral; Vilber Lourmat, Marne la Vallée, France, $\lambda_{max}$ at 365 nm, no emission below 300 nm) at a distance of ~10 cm, or with only one 8 W lamp. To verify that the energy absorbed by the sample was sufficient to give a significant yield of cross-linking, a control reaction was used, based on the coupling, in a closed quartz cuvette, of 4.4 mM benzophenone with 0.1 M diphenylmethanol in benzene (quantum yield of ~0.5−1.0 in degassed solutions; Moore et al., 1961).




In these conditions, and using the 60 W lamp system, the half-life of benzophenone was ca. 18 min.

**Synthesis of the ODN1−Benzophenone Conjugate (ODN2).** ODN1 (30.3 nmol) was dissolved in 180 μL of 0.2 M borate buffer, pH 9. Two milligrams of 4-benzoylbenzoic acid *N*-hydroxysuccinimidyl ester (Sigma; total excess 200×) was solubilized in 20 μL of dry dimethylformamide (distilled on $P_2O_5$). Five aliquots (4 μL each) of this DMF solution were successively added to the stirred ODN solution, at 30 min intervals. The suspension was stirred for another 3 h after the last 4-benzoylbenzoic acid *N*-hydroxysuccinimidyl ester addition (total reaction time 5 h), centrifuged, and the supernatant filtered on a Nylon Acrodisc filter (0.45 μm, Gelman Sciences). This solution was precipitated with ethanol and the insoluble material redissolved in 200 μL of water. Pure ODN2 was isolated from this solution by HPLC (retention time = 25.5 min; yield 63%).

**Synthesis of the Fluorescein−ODN2 Conjugate (ODN3).** ODN2 (9.5 nmol) was dissolved in degassed phosphate buffer (100 mM, pH 7, 95 μL). A solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma) in the same buffer (27.2 μg in 5 μL, 95 nmol, total excess 10×) was added to the ODN solution, and the stirred mixture was allowed to react in the dark under an argon atmosphere for 1 h. Nucleic acids were then precipitated by ethanol in order to eliminate the excess of unreacted TCEP, and the solid phase was dissolved in degassed phosphate buffer (180 μL). A solution of fluorescein-5-maleimide (Molecular Probes, 0.406 mg, 950

nmol, total excess 100×) in acetonitrile:water (1:1; 20 μL) was added to the solution of the reduced ODN2, and the mixture was stirred in the dark under an argon atmosphere for 30 min. ODN3 was first precipitated by ethanol and then purified by HPLC (retention time = 21.5 min; yield = 73%).

**Radiolabeling of ODN3.** Two IodoBeads (Pierce) were placed in an Eppendorf SafeLock tube, washed with phosphate buffer (0.1 M, pH 7.4, 3 × 1 mL), and covered with the same buffer (390 μL). A $Na^{125}I$ solution (10 μL, 1 mCi, Amersham) was carefully added to this suspension under a well-ventilated hood and allowed to react for 5 min. A solution of ODN3 (2.0 nmol) in phosphate buffer (0.1 M, pH 7.4, 100 μL) was then added. After 30 min of reaction, the supernatant was removed and mixed with a solution (500 μL) containing NaI (4.5 mg/mL) and $Na_2S_2O_3$ (4 mg/mL). The excess of radioiodine was eliminated by gel filtration on Sephadex G-25 (NAP-10, Pharmacia). After a concentration step (partial evaporation of the solution in a Speed Vac), an ethanol precipitation allowed one to obtain the radiolabeled ODN3 with a radiochemical purity of nearly 100% [paper chromatography assay on Whatman Chromatography Paper 1, 87 g/m$^2$, 0.16 mm thickness; mobile phase, methanol/water (7:3)].

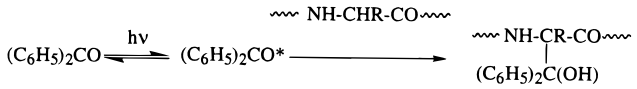
**Photo-Cross-Linking of ODN3 to Human Thrombin.** Human thrombin (176 pmol, ~3000 NIH units/mg of protein, Sigma) was dissolved in PBS−$Ca^{2+}$ buffer (pH 7.4, 578 μL). An aqueous solution of radiolabeled ODN3 (22 μL, 176 pmol) was added. Half of this sample was exposed at 4 °C to UV irradiation in a Petri dish at a total power of 60 W. The other half was incubated in the dark (without being submitted to any UV irradiation). Aliquots submitted to both conditions were taken at different times ranging from 15 to 60 min. These aliquots were analyzed by a 5−15% SDS−PAGE (total amount of thrombin loaded per well = 100 ng). The gel was dried, exposed to a PhosphorImager intensifying screen, and read on a PhosphorImager apparatus (Molecular Dynamics, 410 A model) or autoradiographed.

**Determination of the Photo-Cross-Linking Yield Using a Thrombin Aptamer.** We used in this experiment Tasset's aptamer 60-18[29] [5′-AGT CCG TGG TAG GGC AGG TTG GGG T*GA CT-3′; $K_d$ = 0.5 nM; Tasset et al. (1997)], which bears near the 3′ end an amino-modified T (indicated by an asterisk (*); Amino-Modifier C6 dT, Glen Research), to which a benzophenone moiety was coupled by the protocol used to obtain ODN2 (see above). Two parallel assays were conducted, involving (i) the 3′-amino aptamer 60−18[29] as a negative control and (ii) the 3′-benzophenone-derivatized aptamer 60-18[29]. Either 1.12 nmol of the 3′-amino aptamer 60-18[29] (solubilized in 10 μL of binding buffer, total excess 5×) or the same amount of the 3′-benzophenone-derivatized aptamer 60-18[29] was added to 7.5 μg of human thrombin (224 pmol, ~3000 NIH units/mg of protein, Sigma) solubilized in 740 μL of binding buffer (20 mM Tris acetate, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$; Bock et al., 1992; Macaya et al., 1995). Half of both mixtures was exposed at 4 °C to 60 W UV irradiation in Petri dishes, while the other halves were incubated at 4 °C in the dark. Aliquots were taken at time intervals ranging 15−120 min. These aliquots were analyzed by 5−15% SDS−PAGE (total amount of thrombin loaded per well = 500 ng) and revealed by silver staining.

**Binding Selectivity of ODN3 for Bovine Thrombin.** An aqueous solution of radiolabeled ODN3 (9.2 μL, 73.5 pmol) was added to bovine thrombin (7.3 nmol, ~75 NIH units/mg of protein, Sigma) dissolved in PBS-$Ca^{2+}$ buffer (pH 7.4, 40.8 μL) and incubated at 4 °C in the dark for 2 h, before UV irradiation at 8 W for 15 min. An aliquot of this solution (containing 10 μg of thrombin) was analyzed by a 5−15% SDS−PAGE, and the gel was treated as above.

## RESULTS AND DISCUSSION

**Engineering of the Probe.** To generate an appropriate probe to study ODN−protein interactions, a benzophenone−ODN conjugate was selected for several reasons [reviewed by Dorman and Prestwich (1994)]. (i) Benzophenone is excited at a wavelength (333 nm) that does not affect proteins. (ii) Benzophenone has a broad reactivity: its easily accessible n,π* diradicaloid triplet state is able to cross-link with amino acids residues, one typical reaction being the insertion into the α C−H bond as illustrated below [for a detailed discussion, see Deseke et al. (1998)]:




(iii) Since its unreacted triplet state simply returns to the ground state and does not react with water, benzophenone can be repeatedly excited. Contrary to the irreversibly destructed photophores such as azides, benzophenone derivatives can thus repeatedly absorb photons, until a productive reaction takes place. This property considerably facilitates experiments since benzophenone derivatives can be manipulated in subdued day light without problem. (iv) High yields of photo-cross-linking are usually obtained. Typical photo-cross-linking yields are 40−80% (Williams and Coleman, 1982; Mahmood et al., 1987; Shoelson et al., 1993; Thiele and Fahrenholz, 1993; Chaudhary and Prestwich, 1997). Such clear advantages make benzophenone a molecule of choice in engineering photo-cross-linking ODNs.

The ODN chosen for this study was a PO−ODN 25-mer complementary to the AUG initiation site of the *gag* gene of HIV-1 (Lisziewicz et al., 1994). Its sequence contains only one dG, and the ODN should have no more affinity for thrombin than a random 25-mer. This ODN was further selected because of the wealth of biological information available on its PS counterpart, the gene-expression modulator 91 (GEM 91), an anti HIV-1 former drug candidate from Hybridon (Lisziewicz et al., 1994). We indeed intended to use the probe for subsequent experiments on cells.

The radiolabeling procedure was of course a critical aspect. A standard 5′-$^{32}$P labeling using T4 polynucleotide kinase was not considered, because phosphatases are known to easily remove this label (Shaw et al., 1991). Tritium labeling (Graham et al., 1993) was also disregarded, because of the long exposure times needed to reveal this β-emitting radionuclide. Fluorescein is a phenolic compound able to incorporate the $^{125}$I γ-emitting radionuclide in high yield. Moreover, a fluorescein appendage allows one to use confocal microscopy in experiments involving whole cells (Pichon et al., 1997). The 3′-end of the ODN being reserved for the grafting of benzophenone, fluorescein was attached at its 5′-end. Both 5′- and 3′-ends of our ODN were thus capped by unnatural residues. This protects the sequence against exonuclease degradation (Arar et al., 1995).

**Synthesis and Radiolabeling of the Probe (Figure 1).** The 5′-pyridyl-dithio-3′-amino PO−ODN (ODN1) was obtained by a standard solid-support synthesis. A practi-

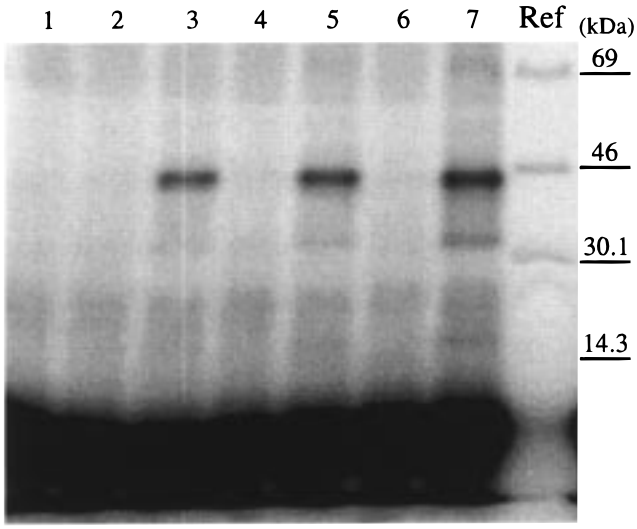



**Figure 2.** Photo-cross-linking of radioiodinated ODN3 to purified human thrombin. [$^{125}$I]ODN3 was incubated with thrombin without preincubation as explained in the text. Lane 1: starting time (0 min, no irradiation performed). Lanes 2, 4, and 6: incubation for 15, 30, and 60 min without irradiation. Lanes 3, 5 and 7: UV irradiation for 15, 30, and 60 min, respectively. Irradiation is necessary for photo-cross-linking to occur. The thrombin-ODN3 complex formed features a $M_r$ of ca. 43 kDa, which corresponds to the sum of the MW of thrombin and ODN3.

cal way to monitor the reactions leading to ODN2 and ODN3 was reversed-phase HPLC. The coupling of ODN1, first with a benzophenone derivative and then with a fluorescein derivative, was in both cases associated with large changes in retention times, allowing for an easy recovery of the pure modified ODNs by semipreparative HPLC.

The grafting of benzophenone on the 3′-$NH_2$ of ODN1 was performed with a suspension of the insoluble 4-benzoylbenzoic acid N-hydroxysuccinimyl ester in an aqueous solution of the ODN. Assays of couplings in several binary solvents were unsuccessful. The chromatogram of the crude mixture was obscured by the presence of several byproducts derived from the acylating agent, but an ethanol precipitation greatly simplified the HPLC trace. Unreacted ODN1 ($t_R$ = 18.1 min) and benzophenone-coupled ODN2 ($t_R$ = 25.5 min, 63%) only subsisted.

The 5′-pyridyldithio moiety of ODN2 was reduced by a water-soluble phosphine. The 5′-HS−ODN was isolated by ethanol precipitation and immediately alkylated by fluorescein-5-maleimide. A new ethanol precipitation was necessary to remove the excess of the alkylating agent. The HPLC trace then showed only a low molecular weight contaminant ($t_R$ = 18.7 min) followed by ODN3 ($t_R$ = 21.5 min, baseline separation). The yield of the synthesis of ODN3 from ODN2 was 73%.

The radioiodination method used in this study was safe and efficient, reaching a specific radioactivity of 16 $\mu$Ci/$\mu$g ODN (160.000 Ci/mol ODN or 18 × $10^{10}$ cpm/$\mu$mol ODN). For comparison, a specific radioactivity of the order of 12 × $10^7$ cpm/$\mu$mol ODN would have been obtained if ODN3 had been tritiated by Graham's method (specific radioactivity accessible with Graham's protocol: ca. 4 × $10^7$ cpm/$\mu$mol of purine; Graham et al., 1993). Sodium thiosulfate was added after radioiodination to quench any remaining electrophilic halogen species. Cold sodium iodide was also added to displace radioactive iodide anions possibly associated to the biopolymer by weak forces. A gel exclusion chromatography, followed by an ethanol precipitation, was then performed.

**Photoaffinity Labeling of Human Thrombin.** As long as no G-quartet is involved in the structure of ODN3, the dissociation constant of its complex with thrombin is estimated to be in the micromolar range or higher: $K_d$ = 8.3 $\mu$M for a random library of 96-mers and 5.0 $\mu$M for

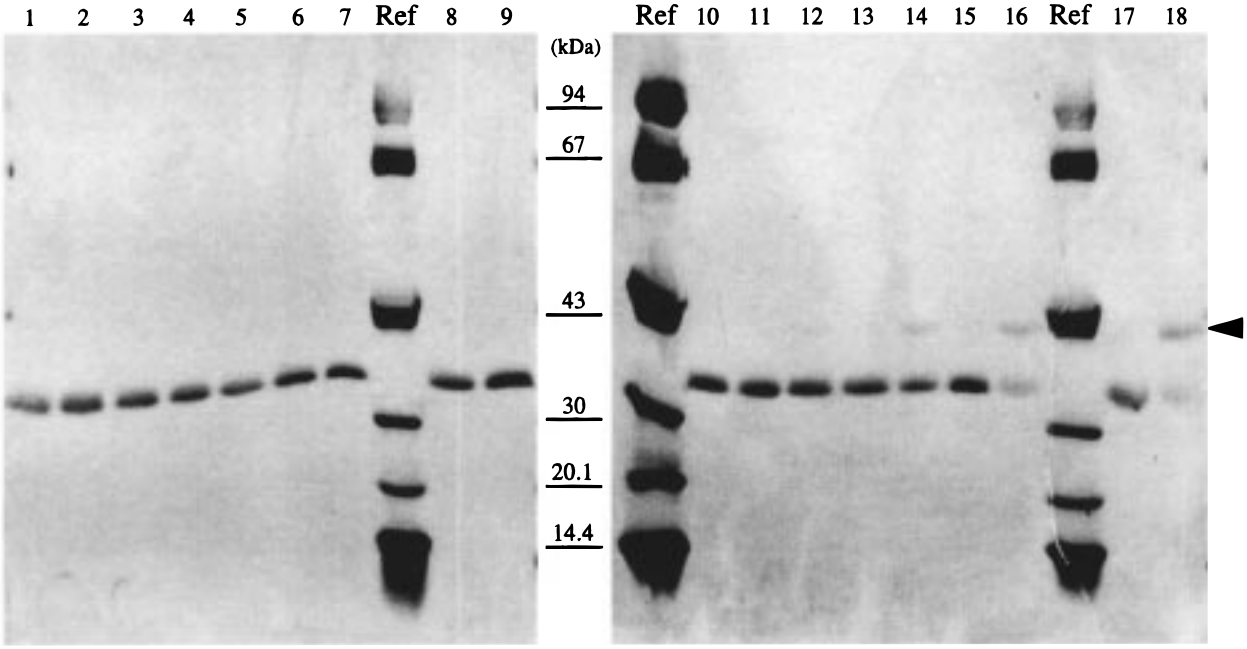



**Figure 3.** Silver-stained PAGE: yield of photo-cross-linking and necessity of a benzophenone residue for the photo-cross-linking to occur. Thrombin aptamer 60−18[29] was incubated with purified human thrombin, without preincubation as explained in the text. On the left, this aptamer was only 3′-protected by an amino function. On the right, the aptamer was bearing a 3′ benzophenone residue. Lanes 1 and 10: starting time (0 min, no irradiation performed). Lanes 2, 4, 6, 8, 11, 13, 15, and 17: incubation for 15, 30, 60 and 120 min without irradiation. Lanes 3, 5, 7, 9, 12, 14, 16, and 18: UV irradiation for 15, 30, 60, and 120 min, respectively. The presence of benzophenone and irradiation were necessary for the photo-cross-linking to occur (the product of which is indicated by an arrowhead at right). The yield of photo-cross-linking, measured by gel scanning, increased from 15% after 15 min of irradiation to 70% after 120 min.

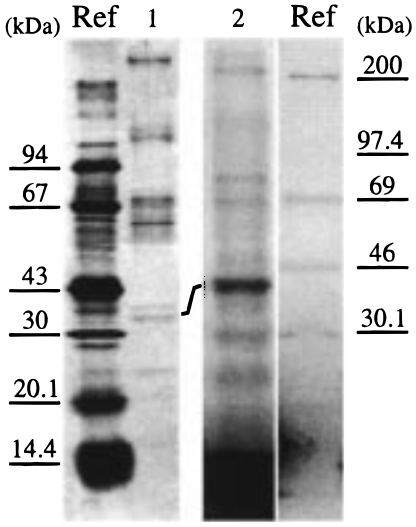



**Figure 4.** Binding selectivity of radioiodinated ODN3 for bovine thrombin in a crude preparation. Lane 1 is a silver staining of 400 ng of a crude bovine thrombin sample. Lane 2 is the PhosphorImager analysis of its photo-cross-linking with [$^{125}$I]ODN3. Markers for silver staining (from Pharmacia Biotech) are shown at left. Radiomarkers (from Amersham) are shown at right. The twisted bar between the two gels indicates band shift upon formation of a covalent complex.

a random library of 66-mers (Tasset et al., 1997); $K_d = 1$ $\mu$M for a 24-mer having a low propensity to form a G-quartet (ODN ***5*** of Macaya et al., 1995). The photo-cross-linking conditions used in this experiment were thus rather demanding as the complexed fraction of the ODN was probably as low as a few percent.

Figure 2 shows the results of the photolabeling of the purest commercially available human thrombin. No labeling was observed without irradiation. The maximum labeling yield was reached within 1 h. The intensity of the thrombin band decreased thereafter (data not shown), probably due to the photohydrolysis of the carbon−iodine bond releasing $^{125}I^-$ iodide anions (Wolf and Kharasch, 1961; Tejedor and Ballesta, 1983; Boule et al., 1984). As expected, the photo-cross-linking of thrombin increased its apparent molecular mass, from ca. 33.5 kDa to ca. 43 kDa, corresponding to the sum of the molecular masses of thrombin and ODN3. The association of ODN3 with thrombin was relatively rapid as a preincubation in the dark before irradiation had no effect on the intensity of the shifted band (data not shown).

**Determination of the Photo-Cross-Linking Yield Using a High-Affinity Thrombin Aptamer.** The irradiation of a dilute equimolar mixture of human thrombin with [$^{125}$I]ODN3 gave a low yield of labeled thrombin, as indicated by the ratio of the protein-associated radioactivity to the radioactivity migrating at the front of the PAGE (assumed to represent unreacted [$^{125}$I]ODN3; Figure 2). This could be due either to the very low equilibrium concentration of the thrombin−ODN3 complex, or to the inefficacy of the coupling reaction. The second hypothesis seemed unlikely, since yields are usually described as excellent. We nevertheless determined the efficacy of photo-cross-linking, using a conjugate of benzophenone with an aptamer featuring a high affinity for thrombin. Tasset's aptamer 60-18[29], whose $K_d$ for thrombin is about 0.5 nM (Tasset et al., 1997), was coupled to a benzophenone molecule, and a mixture of thrombin with an excess of this conjugate was irradiated (in this case, human thrombin was calculated to be practically 100% in the form of its complex). We detected by silver staining on PAGE both unreacted thrombin and the thrombin−ODN covalent complex (Figure 3). The photo-cross-linking yield at a given irradiation time was determined as the amount of complex formed versus the total amount of thrombin. This yield increased with the irradiation time from 15% after 15 min to 70% after 2 h. The efficacy of the photochemical reaction was thus high, as expected.

A benzophenone moiety was necessary for the cross-linking to occur: even under irradiation, the underivatized aptamer formed no detectable SDS-stable complex with thrombin.

**Binding Selectivity of ODN3 for Bovine Thrombin.** As can be seen in Figure 4, ODN3 showed a chemoselectivity for bovine thrombin: despite this protein being only a minor constituent in a crude preparation, the major band observed resulted from the association of ODN3 with bovine thrombin.

**Conclusion and Perpectives.** We reported in this communication an efficient synthesis of a multipurpose ODN derivative. This probe was able to selectively photolabel a low-affinity protein using mild irradiation conditions after such a short time as 15 min. The probe was designed for time-resolved and space-resolved experiments on whole cells. We are currently using this tool for deciphering the process of ODN entry into mammalian cells.

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