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The Application of an Immobilized Molecular Beacon for the Analysis of the DNA Binding Domains from the Ecdysteroid Receptor Proteins Usp and EcR's Interaction with the *hsp27* Response Element

TOMASZ KRUSIŃSKI,¹ ANNA LASKOWSKA,² ANDRZEJ OŻYHAR,¹ and PIOTR DOBRYSZYCKI¹

The nonstandard molecular beacon described in this article consists of 2 fragments, each built of a short single-stranded oligonucleotide sequence and a double-stranded sequence. One of these hybridization probes, labeled with a fluorescence donor (fluorescein), is solid phase immobilized. The second nonimmobilized probe is labeled with a fluorescence quencher (dabcyl). Annealing of both probes via single-stranded sequences was possible only in the presence of a specific protein molecule that recognized the response element sequence initially separated between the immobilized and nonimmobilized fragments. The system was applied successfully to detect the sequence-specific interaction of a natural *hsp27* response element from the promoter of the *hsp27* gene with the DNA binding domains of 2 nuclear receptor proteins: ultraspiracle Usp (UspDBD) and the ecdysone receptor EcR (EcRDBD). Measured in the absence of EcRDBD, the dissociation constant, K_d of the UspDBD-*hsp27* complex, was determined to be 3.26 nM, whereas for UspDBD devoid of the A-box (UspDBD Δ A-*hsp27*), the dissociation constant was 4.81 nM. The respective K_d values in the presence of EcRDBD were 2.43 nM and 10.80 nM. The results obtained with the immobilized molecular beacon technology were in agreement with those obtained by conventional fluorescence titrations and by fluorescence resonance energy transfer measurements with nonimmobilized beacons. (*Journal of Biomolecular Screening* 2008:899-905)

Key words: ecdysone receptor, ultraspiracle, FRET, molecular beacon, immobilization

INTRODUCTION

THE BINDING AFFINITY OF DNA-BINDING PROTEINS to target DNA sequences shows how individual amino acids contribute to specific interactions with response elements. For some time, we have been interested in introducing molecular beacon technology into the quantitative analysis of the interaction of an ecdysteroid receptor with a natural pseudo-palindromic response element from the *hsp27* gene promoter. Typically, molecular beacons are oligonucleotide probes that can identify the presence of specific nucleic acids in solution.¹

The idea of using molecular beacons to detect DNA protein binding activity appeared a few years ago.^{2,3} The central feature of the assay is the protein-dependent association of 2 DNA fragments, each containing about half of the DNA sequence that defines the protein binding site. To obtain insight into the nature of the formation of the UspDBD-*hsp27* complex, a prerequisite to subsequent building up of the whole UspDBD/EcRDBD-*hsp27* complex, we previously demonstrated quantitatively interaction of UspDBD with the *hsp27* sequence using fluorescence measurement-based methodologies.⁴ The results obtained with molecular beacons were compared with those obtained with the common method of the fluorescence anisotropy. Both techniques applied allowed us to obtain consistent binding constants for protein-DNA complex formation.

The aim of this study was to use an immobilized molecular beacon system to demonstrate protein-dependent association of DNA fragments, as in the case of the unimmobilized system, by measuring the dissociation constants of the UspDBD/EcRDBD-*hsp27* complexes. This article introduces a rapid, simple, and sensitive fluorescence assay for detecting DNA binding proteins. To the best of our knowledge, this is the first time an immobilized molecular beacon has been used to detect protein

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activity, although the idea was presented originally.³ Preliminary results with a nonimmobilized system have been published recently.⁴ We have developed an approach that uses a surface-immobilized biotinylated fragment of *hsp27* labeled with a fluorescence donor probe. DNA sequences were chosen in such a way that the molecular beacon hybridizes to adjacent regions only in the presence of protein molecules. The assay involves the coassociation of DNA fragments that recognize 2 proteins, UspDBD and EcRDBD. The DNA duplexes contain short fluorophore- or quencher-labeled, complementary oligonucleotides. One duplex is attached to a solid phase by a non-DNA linker. The protein-induced association of the DNA fragments, containing the previously divided response element *hsp27*, results in bringing the 2 “signaling” oligonucleotides into proximity, which then quenches the donor molecule fluorescence signal as a result of the resonance energy transfer between the donor and the acceptor probes. The UspDBD/EcRDBD-*hsp27* complex was used as a model system to provide justification for using a molecular beacon design in biosensors. The molecular beacon was applied for the first time in a biochip for detecting and analyzing the breast cancer gene *brca1*.⁵ However, the detection system was limited to recognizing the loop and stem structure of a short oligonucleotide.

MATERIALS AND METHODS

Molecular beacon preparation

The following oligonucleotides (MWG Biotech AG, Ebersberg, Germany) were used to prepare the molecular beacons:

Biotin-TEG-5'-CTGGCATATAGCGACA-3' (01),
D-5'-TGGGTTCAATGCACT TGTC CAATGAA-3' (02),
5'-TTCATTGGACAAGTGCA-3' (03), and
5'-TTGAA CCCA FGTGCTATATGCCAG-3' (04),

where **F** denotes the dT-fluorescein, **D** denotes dabcyl-5'-dT, and **TEG** denotes the tetra ethylene glycol linker.

01 and 04 hybridization formed DNA duplex 1 (D1), labeled with a fluorescence donor - fluorescein, containing a 9-base overhang.

Biotin-TEG-
-5'CTG GCA TAT AGC GAC A-3'
3'-GAC CGT ATA TCG CTG FAC CCA AGT T-5' (D1)

02 and 03 formed DNA duplex 2 (D2) with a complementary overhang, labeled with a dabcyl - non-fluorescent energy acceptor (quencher).

5'-D GGG TTC AAT GCA CTT GTC CAT-3'
3'-A CGT GAACAG GTA-5' (D2)

To obtain D1 or D2 duplexes both, we mixed 01-04 and 02-03 oligonucleotide pairs with a 1- μ M concentration in a 50-mM Tris buffer, 100 mM NaCl, 5 μ M ZnCl₂, and 1 mM 2-mercaptoethanol, pH 7.8, at 25 °C, then heated to 96 °C for 1 min and cooled to room temperature over 2.5 h. The coupling of D1 and D2 reconstructs the DNA duplex (D1:D2), which contains the following *hsp27* response element sequence:

Biotin-TEG-5'-CTGGCATATAGCGACADGGGTTCAATG-
3'-GACCGTATATCGCTG FACCCAAGTTAC-

-CACTTGTCCAATGAA-3'
-GTGAACAGGTTACTT-5'

The ecdysteroid receptor binding site is described in italics.

Oligonucleotide concentrations were calculated from the absorption spectra of oligonucleotide solutions recorded from 200 to 600 nm, using 177,440 M⁻¹cm⁻¹ (01), 280,910 M⁻¹cm⁻¹ (02), 187,190 M⁻¹cm⁻¹ (03), and 259,400 M⁻¹cm⁻¹ (04) extinction coefficients.^{6,7} Absorption spectra were recorded with Cary 300 UV-VIS (Varian, Inc., Palo Alto, CA) and Ultrospec 4000 UV/VIS (Pharmacia Biotech, Piscataway, NJ) spectrophotometers. The oligonucleotide probes were designed to be complementary as in the natural *hsp27* regulatory element (see **Fig. 1A**).

Protein preparation

The expression and purification of the UspDBD and the EcRDBD proteins were performed as described previously⁸ with a slight modification.⁹

Immobilization of the biotin-modified fragment of the molecular beacon

The biotin-modified D1 DNA duplex was immobilized on a black 96-well microplate coated with covalently bound streptavidin (Streptavidin Immobilizer™, Nunc, Roskilde, Denmark). After rinsing the plate 3 times with 300 μ L 5xSSCT buffer (75 mM sodium citrate, 750 mM NaCl, 0.05% (v/v) Tween-20, pH 7.0, at 25 °C) per well, 100 μ L of a 10-nM solution of biotin-tagged D1 in a 5xSSCT buffer was added, and the plate was incubated overnight at 26.5 °C. Then the wells were washed 3 times with 300 μ L of a 2xSSCT buffer (30 mM sodium citrate, 300 mM NaCl, 0.05% (v/v) Tween-20, pH 7.0, at 25 °C). The design of the array, based on a molecular beacon, is shown in **Figure 1B**. The probe sequences were chosen such that the D1 and D2 duplexes hybridize only in the presence of UspDBD and/or EcRDBD proteins.

Fluorescence measurements

To each well containing a D1-immobilized duplex (prepared as described above), 100 μ L of the D2 dabcyl-labeled duplex

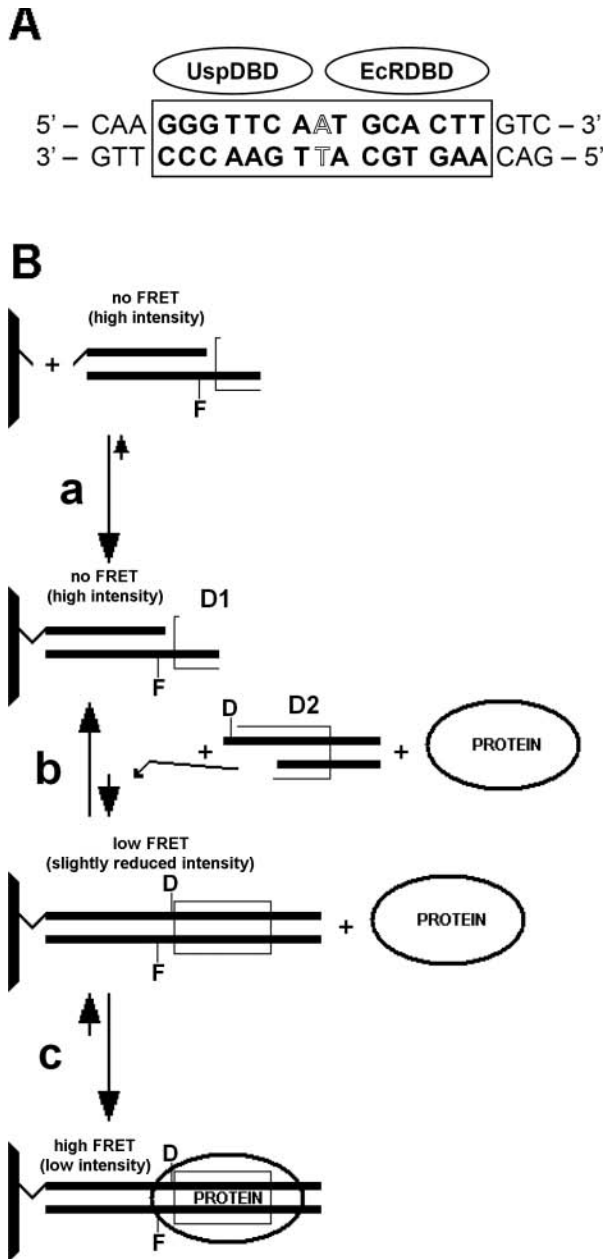


FIG. 1. (A) The *hsp27* ecdysone response element sequence. (B) Overall design of the immobilized molecular beacon for sequence-specific DNA binding proteins: a—D1 fluorescein (donor)-labeled duplex immobilization on a streptavidin-precoated well surface, b—the protein-independent association of D1 with D2 dabcyl (acceptor)-labeled duplex, and c—D1-D2 association in the presence of the DNA-binding protein; F denotes the fluorescein, D-dabcyl. FRET, fluorescence resonance energy transfer.

was added with an appropriate amount of protein samples with a concentration range of 0 to 500 nM and incubated for 75 min at room temperature. Measurements were performed with a Victor² fluorometer (PerkinElmer, Waltham, MA) using F485

extinction and F535 emission filters and the stabilized energy lamp control method at 25 °C.

Fluorescence measurements for the same nonimmobilized sequences were performed under identical conditions as with the immobilized DNA fragments in a 115F-QS quartz cuvette (Hellma GmbH & Co. KG, Müllheim, Germany) with a FLU-OROLOG-3 fluorometer (Spex, Jobin Yvon, Inc., Longjumeau, France) using an excitation and emission wavelength of $\lambda_{EX} = 497$ nm and $\lambda_{EM} = 518$ nm, respectively.

All measurements were performed in a 50-mM Tris buffer, 100 mM NaCl, 5 μ M ZnCl₂, 1 mM 2-mercaptoethanol, pH 7.8, at 25 °C.

Data analysis

Molecular beacon methodology was used to determine the dissociation constant through a 2-step approach: (1) titrations of a donor-labeled DNA half-site with an acceptor-labeled DNA half-site and (2) titrations of donor- and acceptor-labeled DNA half-sites with a protein as described previously.^{2,4,10} Data points were analyzed according to equations (1) and (2) for the first and the second steps, respectively:

$$F_1 = F_{MAX} + (F_{MIN} - F_{MAX}) \cdot \left[\frac{(K_1 + [D1]_T + [D2]_T)}{2[D1]_T} + \frac{\sqrt{(K_1 + [D1]_T + [D2]_T)^2 - 4[D1]_T \cdot [D2]_T}}{2[D1]_T} \right] \quad (1)$$

and

$$F_1 = F_{MAX} + (F_{MIN} - F_{MAX}) \cdot \left[\frac{(K_{app} + [D1]_T + [P]_T)}{2[D1]_T} + \frac{\sqrt{(K_{app} + [D1]_T + [P]_T)^2 - 4[D1]_T \cdot [P]_T}}{2[D1]_T} \right] \quad (2)$$

where F is the observed fluorescence intensity; F_{MAX} is the maximal value of fluorescence intensity; F_{MIN} is the fitted value of the minimal fluorescence intensity; K_1 is the dissociation constant of the D1:D2 complex; K_{app} is the apparent dissociation constant of the DNA-protein complex; and $[D1]_T$, $[D2]_T$, and $[P]_T$ are the total concentrations of the D1, D2 half-sites and protein, respectively. Taking into account the reaction scheme $D1: D2 + P \rightleftharpoons D1: D2: P$ and the calculated values of K_1 and K_{app} , one can obtain the K_d constant according to equation (3):

$$K_d = \frac{K_{app}}{K_1} \cdot [D2], \quad (3)$$

where $[D2]$ is the equilibrium concentration of the D2 half-site.

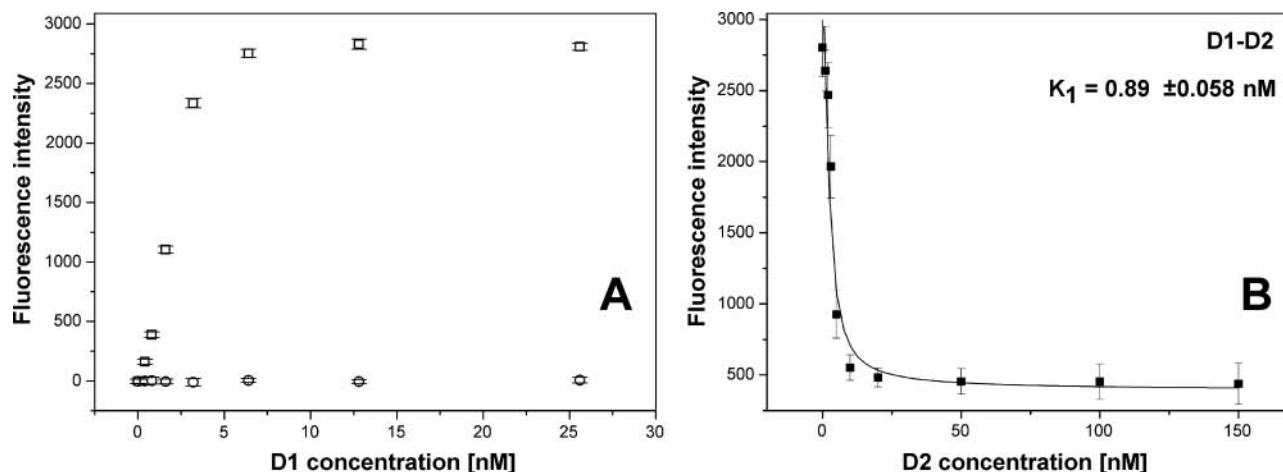


FIG. 2. (A) Determination of the amount of the fluorescein-labeled D1 duplex, immobilized on a streptavidin-coated well surface after rinsing 3 times (open squares), and for the non-biotinylated analog of the D1 duplex after rinsing 3 times (open circles). (B) Estimation of the dissociation constant (K_1) of the D1-D2 complex. Titration curve of the immobilized 0.3-pmol D1 duplex with increasing amounts of the D2 duplex. Measurements were performed in a 50-mM Tris buffer, 100 mM NaCl, 5 μ M ZnCl₂, 1 mM 2-mercaptoethanol, pH 7.8, at 25 °C. Error bars indicate standard deviation values of 3 measurements.

Preparation of the detection plate

Based on the data obtained from the calibration curve $y = 1001x - 203$ (x denotes D1 concentration in nM) and saturation curve of the D1 binding to the microplate surface, it was assumed that each well binds 0.3 pmol of the biotinylated D1 duplex (which corresponds to 100 μ L of the 3-nM solution; **Fig. 2A**). Therefore, we let the D2 duplex concentration be up to 150 nM (50 times higher) for K_1 determination (D1-D2 coupling in the absence of protein) and 3.25 nM for K_{app} determination (D1-D2 coupling in the presence of protein).

We have done a set of control experiments to show specificity of the biotinylated DNA to the plate surface by measuring the fluorescence intensity of increasing the D1 duplex and its nonbiotinylated analog concentration, which were incubated overnight on a microplate coated with streptavidin. The solutions were then removed from the microplate, and wells were rinsed 3 times with a buffer to remove the unbound duplexes. Next, the wells were filled with the buffer, and again the fluorescence intensity was measured. The fluorescence signal was observed only in wells where the biotinylated duplex was initially incubated. The curve shown in **Figure 2A** (empty squares) supports the thesis of specific binding of the biotinylated D1 duplex to the plate surface. According to the plate producer (Nunc), total binding capacity for free biotin is as follows: 20 pmol/well in a 96-well (coating volume 100 μ L), whereas in our experiments, it was 0.3 pmol/well, probably due to the size of the biotin-labeled D1 molecule. In the presence of the D2-dabcyl duplex (fluorescence quencher), the initial fluorescence intensity decreased from ca. 2800 to 2100 (compare

the initial points in **Fig. 2B** and **Fig. 3**), which we believe constitutes a substantial change. In addition, we have shown the absence of D2 duplex binding to the plate surface by comparing the degree of fluorescence quenching of nonimmobilized D1 titrated with D2, where D2 was incubated overnight (1) on a plate or (2) in an Eppendorf tube. Both samples were quenched $16.5\% \pm 1.5\%$. Moreover, nonspecific protein binding to the microplate was not observed (not shown). Absorbance values measured at 280 nm of the UspDBD protein sample, incubated on the microplate for 75 min and incubated in the Eppendorf tube, did not differ in the measurement error range.

RESULTS AND DISCUSSION

Dissociation constant estimation

We have designed a molecular beacon to study ecdysone receptor and its interaction with the *hsp27* response element by attaching fluorescence donor and quencher probes to separated DNA fragments. Conditions for the experiments for a protein-induced DNA hybridization in the presence of UspDBD and/or EcrRDBD have been described. The detection system uses changes in the fluorescence intensity from the resonance energy transfer between pairs of fluorophores. Unlike the typical fluorophore-quencher beacon design, the separation of the response element into fragments makes it possible to visualize the hybridized state of the beacon in the presence of UspDBD and the unhybridized state of the beacon in the absence of the protein. The system allows us to quantify the formation of the protein-DNA complex. In the first step of the presented methodology (see

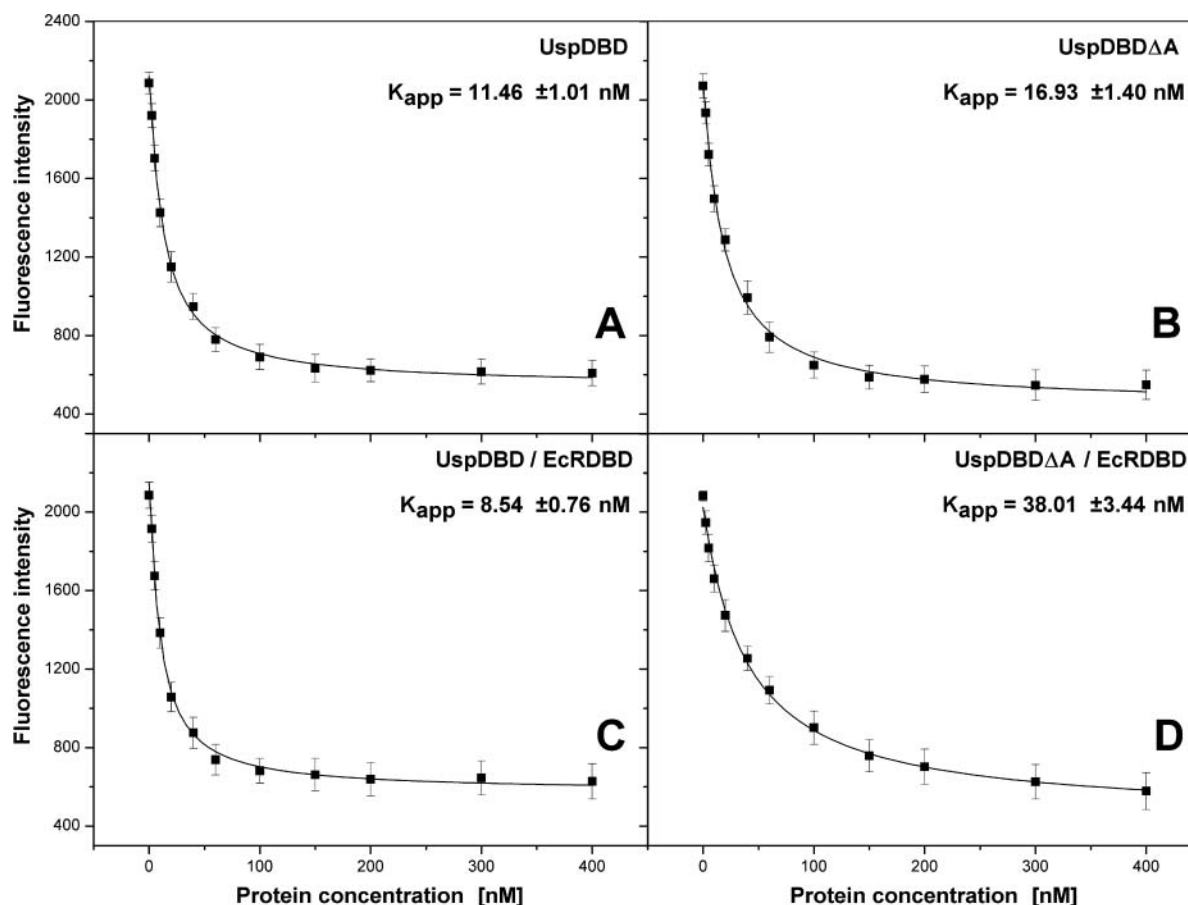


FIG. 3. Protein-D1-D2 complex dissociation constants estimation (K_{app}) using the immobilized molecular beacon. Titration curves of the immobilized 0.3-pmol D1 duplex in the presence of 0.325 pmol of the D2 duplex with appropriate amounts of (A) UspDBD, (B) UspDBD Δ A, (C) UspDBD in the presence of an equimolar amount of EcRDBD, and (D) UspDBD Δ A in the presence of an equimolar amount of EcRDBD. Measurements were performed in a 50-mM Tris buffer, 100 mM NaCl, 5 μ M ZnCl₂, 1 mM 2-mercaptoethanol, pH 7.8, at 25 °C. Error bars indicate standard deviation values of 3 measurements.

Fig. 1B), the immobilized D1 duplex was titrated with a significant excess of D2 oligonucleotide (Fig. 2B). The nucleotide sequence of the D1 duplex was longer than the one used in a recent publication⁴ to make a longer distance between the point of immobilization and the response element sequence, which consequently enhanced the flexibility of the oligonucleotide. Numerical fitting of data points to equation (1), as described previously,⁴ gave a K_1 value of 0.89 ± 0.06 nM. In the control experiment, the value of 4.69 ± 0.61 nM was obtained using nonimmobilized reagents (data not shown). In the second step of titration, increasing concentrations of UspDBD (0–400 nM) were added to constant amounts of the D1 and D2 duplexes (Fig. 3A). Data points were fitted again to equation (2), and the K_{app} value was 11.46 ± 1.01 nM. Finally, the $hsp27$ -UspDBD complex dissociation constant, $K_d = 3.26 \pm 0.50$ nM, was calculated from K_1 and K_{app} according to equation (3).

All 12 amino acids from the C-terminal extension of Usp correspond to RXR's T-box, and 5 of the 10 amino acids correspond

to the A-box.¹¹ As previously shown, using an electrophoretic mobility shift assay (EMSA), deleting 14 amino acids from the region of UspDBD corresponding to the A-box (UspDBD Δ A) did not significantly change the $hsp27$ binding properties in comparison with the wild-type protein.⁸ The interaction of UspDBD Δ A with $hsp27$ was analyzed under identical conditions as with full-length UspDBD (Fig. 3B). K_{app} was 16.93 ± 1.40 nM, whereas K_d was 4.81 ± 0.71 nM. Similarly, to examine the influence of EcRDBD on UspDBD binding to $hsp27$ (Fig. 3C,D), we performed titrations of constant amounts of D1 and D2 duplexes with equimolar amounts of the 2 proteins. K_{app} and K_d values obtained in the presence of EcRDBD, for the $hsp27$ -UspDBD complex, were 8.54 ± 0.76 nM and 2.43 ± 0.37 nM, respectively, whereas for the $hsp27$ -UspDBD Δ A complex, values were 38.01 ± 3.44 nM and 10.80 ± 1.67 nM, respectively.

It was not possible to reach saturation for the D1 and D2 duplexes titrated with the T-box mutant UspDBDV71A, even at a high UspDBDV71A concentration, so it was not reasonable

to quantitatively describe binding from the experimental points (data not shown). One can conclude that there is only weak protein-DNA interaction in comparison with wild-type UspDBD. This result is in agreement with the data obtained for UspDBD and EcRDBD proteins, which shown with the qualitative EMSA technique that the single-point mutation of Val-71 for alanine significantly decreases affinity to the *hsp27* element. Moreover, CD spectra of V71A mutants have reduced ellipticity compared with the wild-type proteins, indicating that alanine substitution lowered the content and possibly altered distribution of the secondary structure elements.^{12,13}

The model described in this article employs 2 equilibria linked between (1) 2 complementary DNA fragments and (2) a protein and the DNA regulatory element. Equilibrium titration experiments provide evidence supporting the model described² for a nonimmobilized system and also show that it is sufficient to describe the protein-DNA interaction for immobilized DNA fragments.

Our research demonstrates the application of an immobilized molecular beacon for determining the binding parameters of the *hsp27*-UspDBD/EcRDBD complex. Results obtained with immobilized molecular beacons are in agreement within an error range of those obtained previously for a nonimmobilized system or with the results obtained applying steady-state fluorescence anisotropy measurements (Table 1).⁴ Thus, independent of the method used, the deletion of the A-box in UspDBD has a small but reproducible effect on protein binding to the response element. On the other hand, replacement of Val-71 for Ala in the T-box causes a dramatic decrease of UspDBD affinity to the *hsp27* element, confirming the role of Val-71 for the proper recognition and binding to the response element. The data presented in Table 1 indicate that in the presence of EcRDBD, one can observe an increase of UspDBD affinity to *hsp27*. This result suggests cooperative binding of UspDBD in solution. Removal of the A-box in the presence of EcRDBD causes considerable weakness of the binding because of the nonideal fit of both proteins to the response element.

The described interaction of the ecdysone receptor DNA binding domains with the response element is associated with the 5' half-site of *hsp27* because the point of separation (cleavage) of the DNA fragments was located on the 5' side of the *hsp27* element. The 5' half-site is preferentially bound by both receptor components, but UspDBD's affinity is ca. 4 times greater than that of EcRDBD.⁸ Thus, we conclude from the dissociation constants measured in the absence of EcRDBD that the obtained value resulted from UspDBD's binding rather than from the EcRDBD binding to the 5' half-site. The results presented in this article are in agreement with the previously published qualitative data obtained with the EMSA method.^{8,14}

In this article, we have reported a sensitive fluorescence molecular beacon assay for the detection of DNA binding proteins UspDBD and EcRDBD. The central feature of the assay is the protein-dependent association of 2 DNA fragments. One

Table 1. Comparison of the Results Obtained With a Molecular Beacon Immobilized on a Microplate and in Solution

Complex	K_d [nM]		
	Immobilized Molecular Beacon	Nonimmobilized Molecular Beacon ^d	Fluorescence Anisotropy ^d
<i>hsp27</i> -UspDBD	3.26 ± 0.50	1.42 ± 0.48	1.30 ± 0.36
<i>hsp27</i> -UspDBD-EcRDBD	2.43 ± 0.37	Not determined	Not determined
<i>hsp27</i> -UspDBDΔA	4.81 ± 0.71	6.60 ± 0.50	6.65 ± 0.59
<i>hsp27</i> -UspDBDΔA-EcRDBD	10.80 ± 1.67	Not determined	Not determined
<i>hsp27</i> -UspDBDV71A	Not detected	Not detected	Not detected

Dissociation constants obtained for *hsp27*-UspDBD complexes obtained in the presence and absence of EcRDBD. The DNA duplex containing the response element half-site was immobilized on biotinylated microplates. Standard errors were calculated according to Origin 7.5 software (OriginLab) based on data from 3 measurements.

DNA duplex is solid phase immobilized. Although further studies are required to evaluate the capabilities of an immobilized molecular beacon in sensor systems, the results presented in this study illustrate the potential of the molecular beacon in biosensors as a precise tool for the quantitative detection of the interaction between response elements and their respective transcription factors. The type of design used in this work is suitable for simultaneous detection of hundreds of DNA target sequences in one step using a series of beacons immobilized on a solid phase. The results are quantitative, and the dissociation constants are comparable in error range to the data obtained with conventional fluorescence measurements.

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