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ORIGINAL PAPER

Equilibrium Analysis of the DNA Binding Domain of the Ultraspiracle Protein Interaction with the Response Element from the *hsp27* Gene Promoter—the Application of Molecular Beacon Technology

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Abstract Ecdysteroids initiate molting and metamorphosis in insects via a receptor which belongs to the superfamily of nuclear receptors. The ecdysone receptor consists of two proteins: the ecdysone receptor (EcR) and the ultraspiracle (Usp). The EcR–Usp dimer conducts transcription through a $hsp27_{pal}$ response element. Usp acts as an anchor orienting the whole complex on the DNA. The molecular beacon methodology was applied to detect the sequence-specific DNA of a natural $hsp27_{pal}$ or mutated protein interaction with the DNA binding domain from the Usp. The dissociation constant, K_d, of the UspDBD–$hsp27_{pal}$ complex was determined to be 1.42±0.48 nM, whereas K_d for $UspDBD_{\Delta A}$–$hsp27_{pal}$ was 6.6±0.5 nM. Mutation of Val-71 for Ala blocks formation of the protein-DNA complex in contrast to Glu-19 mutation for Ala for which K_d=4.31±1.01 nM. The results obtained with the molecular beacon technology are related to those obtained by fluorescence anisotropy titrations.

Keywords Ecdysteroid receptor · Ultraspiracle · Fluorescence anisotropy · Molecular beacon · *Drosophila melanogaster*

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Abbreviations

DBD	DNA binding domain
20E	20-hydroxyecdysone
$hsp27_{pal}$	20-hydroxyecdysone response element consisting of an imperfect palindrome from the promoter region of the *Drosophila hsp27* gene
RXR	retinoid X receptor
Usp	product of the *ultraspiracle* gene
UspDBD	Usp DNA binding domain
EcR	product of the *EcR* gene
FRET	fluorescence resonance energy transfer
EMSA	electrophoretic mobility shift assay

Introduction

The steroid hormone, 20-hydroxyecdysone, induces the molecular events that lead to molting and metamorphosis in insects and crustaceans. The effects of 20E are mediated by a member of the nuclear receptor superfamily, a product of the *EcR* gene. Although the EcR can bind 20E on its own, binding is greatly stimulated by its heterodimerization partner, a product of the *ultraspiracle* gene (Usp), which is another member of the nuclear receptor superfamily. Interestingly, Usp exhibits a strong structural and functional similarity to a vertebrate retinoid X receptor (RXR) [1]. An intriguing feature of the EcR/Usp heterodimer, distinguishing it from vertebrate counterparts, which tend to form heterodimeric complexes on inherently asymmetric DNA-binding sites composed of directly repeated half-sites, is its propensity for response elements arranged as pseudopalindromes with a single intervening nucleotide. Our mutational studies of the interaction of the Usp and EcR DBDs (UspDBD and EcRDBD, respectively) with the pseudo-

palindromic response element from the *hsp27* gene promoter ($hsp27_{pal}$) have demonstrated that the half-sites of this element contribute differently to the binding of the UspDBD/EcRDBD heterodimer components; the 5′ half-site exhibited higher affinity for both DBDs than the 3′ half-site. This observation—along with the EMSA data demonstrating that the UspDBD exhibits ca. fourfold higher affinity toward the 5′ half-site than EcRDBD—suggested that the interaction of the UspDBD with this half-site is a key factor dictating the polarity of the complex (5′-UspDBD-EcRDBD-3′). This polarity has been verified by the crystal structures of the UspDBD/EcRDBD complexes with an idealized non-natural element organized as an inverted repeat of the 5′-AGGTCA-3′ sequence separated by 1 bp [2]. It has also been very recently verified with the natural regulatory element from the promoter of *hsp27* gene [3].

To obtain insight into the nature of the thermodynamic forces involved in the formation of the UspDBD–$hsp27_{pal}$ complex, a prerequisite to the subsequent building of the whole UspDBD/EcRDBD–$hsp27_{pal}$ complex, we decided to quantitatively analyze interaction of UspDBD to the $hsp27_{pal}$ sequence using fluorescence-measurement based methodologies. Firstly, we decided to use a molecular beacon methodology. This modern technique was adapted for measuring the sequence-specific DNA-binding activity of proteins [4]. The assay is rapid, sensitive, selective and nonradioactive as an EMSA test and easy to apply in biosensors [5]. In the second step the results obtained with molecular beacons were compared with those obtained with the common method of fluorescence anisotropy. Both applied techniques allowed us to obtain consistent binding constants for protein–DNA complex formation.

Detailed analyses of the interaction of the EcR and Usp DNA-binding domains (DBDs) with the pseudo-palindromic response element from the *hsp27* gene promoter showed that UspDBD acts as a specific anchor that preferentially binds the 5′ half-site of this element and thus locates the complex in the defined orientation [6–8].

The functional and structural similarities of vertebrate RXRs and Usp are very interesting because of evolutionary implications and the possibility of Usp engagement in the coordination of various hormonal signals [9].

Steady-state fluorescence measurements are widely used for the analysis of protein–ligand, protein–protein, and protein–DNA interactions. In particular, fluorescence anisotropy is a convenient parameter, because it is sensitive to the changes of molecule dimension and/or shape, fluorophore microenvironment and mobility [10], e.g., anisotropy measurements were successfully used for the investigation of the tryptophan repressor (TrpR) with a 25 bp oligonucleotide containing the Trp operator sequence [11]. According to the proposed model, the values of the dissociation constants (K_d) for the protein dimer, tetramer and octamer binding were calculated as 0.13 nM, 12.5 nM and 2 μM, respectively. Fluorescence anisotropy measurements gave a lot of data for understanding the specificity of the interaction of the glucocorticoid receptor with DNA [12]. We analyzed the affinity between the regulatory element (GRE) and the wild type of DNA binding domain ($GRDBD_{wt}$) and the protein triple mutant ($GRDBD_{EGA}$). $GRDBD_{wt}$ preferentially binds the GRE palindromic consensus sequence (K_d=1.3 μM), while $GRDBD_{EGA}$ prefers the estrogen response element (ERE) (K_d=4.5 μM). Cooperativity of the human estrogen receptor α (ER) binding to the specific DNA sequence was analyzed [13]. Fluorescein-labeled 35 bp oligonucleotides were used for the thermodynamic analysis of the contribution of a particular bp in the protein–DNA complex formation. The interaction energy between ER and its' target sequence is nearly equally distributed along the binding element. The structural basis of RXR–DNA interaction with fluorescence anisotropy measurements was described [14]. The DNA binding domain of 9-cis retinoic acid receptor binds a specific consensus sequence of a direct repeat type with one or two bp spacers (DR1 or DR2, respectively). RXR DBD binds DR1 with K_d=0.37 μM, while DR2 with K_d=0.5 μM. The results described above were obtained with this relatively simple method which is not always possible to apply, because the overall signal change on protein binding is often too small.

A new methodology for detecting DNA binding proteins has recently been described with a physical model (see Fig. 1) which allows quantitative analysis of the protein–DNA interaction [4, 15]. The assay is based on the hybridization of two DNA fragments, each labeled with a fluorescence dye, one with a donor molecule and a second with an acceptor fluorophore, often a fluorescence quencher, resulting in energy transfer. The pair of DNA fragments associates in a protein-dependent manner, because a beacon splits the DNA sequence that defines the protein binding site. Moreover, DNA fragments contain short complementary overhangs which introduce little tendency for the fragments to associate.

Materials and methods

The fluorescently labeled DNA for anisotropy measurements

The DNA fragments with sequences presented in Table 1. used in this study were obtained from Eurogenetec S.A. (Belgium), Biomers (Germany) and Genosys (Sigma-Genosys, Ltd., UK).

The dye fluorescein (FL) was coupled to the 5′ end of the strand via a C_6 linker. The double-stranded DNA was

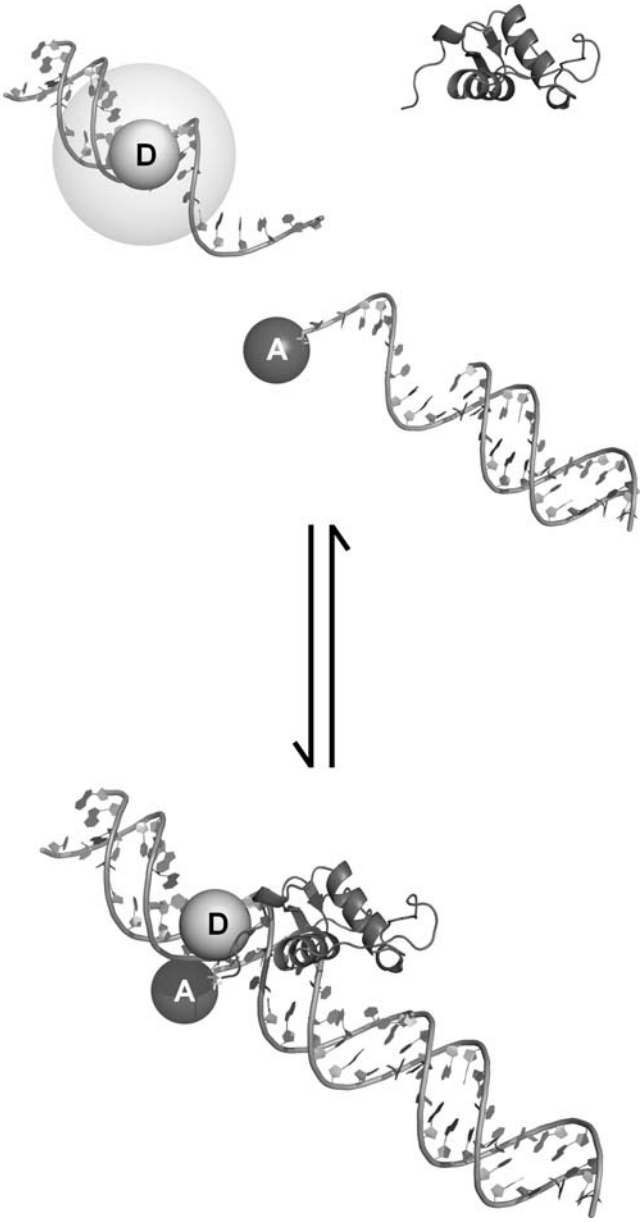

Fig. 1 Design of the molecular beacon. Protein-induced DNA half-sites annealing. Fluorescein (**D***onor*) emission is quenched by dabcyl (**A***cceptor*), which is in close proximity after complex formation, resulting in a high FRET between probes

annealed by hybridizing equal amounts of single-stranded oligonucleotides in 50 mM Tris, 100 mM NaCl, 10% (v/v) glycerol, 5 μM $ZnCl_2$ (pH 7.8) with overnight cooling from 95 to 20 °C. The following 21 bp long DNA molecules were obtained:

(1) Sequence containing *hsp27* response element (*hsp*27_{pal}—signed with bold letters)—O1–O2:

```
          ---   --- -++  +++ +++
          765   432 101  234 567
5' - FL - CAA GGG TTC AAT GCA CTT GTC - 3'
3' - GTT  CCC AAG TTA CGT GAA CAG - 5'
```

(2) $hsp27_{pal}$ sequence mutated at −5T and −6T positions ($hsp27_{mut}$)—O3–O4:

```
5' - FL - CAA GTT  TTC AAT GCA CTT  GTC - 3'
3' - GTT  CAA AAG TTA CGT GAA CAG - 5'
```

(3) Non-specific sequence containing the same amount of GC bp as $hsp27_{pal}$, but random sequence ($hsp27_{ran}$)—O5–O6:

```
5'- FL - CGC TTT  GGA CAG TAC ACA GAT - 3'
3' - GCG AAA CCT GTC ATG  TGT  CTA - 5'
```

Absorption spectra of these solutions were recorded from 240 to 600 nm to determine the concentration of the samples and to control the quantity of labeling. The DNA absorption coefficients used were ε_{260}=234,631 M^{-1} cm^{-1} (O2), ε_{260}=250,794 M^{-1} cm^{-1} (O4), ε_{260}=228,070 M^{-1} cm^{-1} (O6), all estimated from a weighted average of the mononucleotide extinction coefficients, according to a method described by the oligonucleotide producer (www.genosys.com). Concentrations of fluorescently labeled oligonucleotides were determined using ε_{494}=65,000 M^{-1} cm^{-1} for fluorescein [16] with:

$$c = \frac{A_{260} - 0,35 \cdot A_{494}}{\varepsilon \text{ ds oligo}} \quad (1)$$

where $\varepsilon_{\text{ds oligo}}$=256,540 M^{-1} cm^{-1}, A_{260} and A_{494} absorbance was measured at 260 and 494 nm, respectively. The degree of labeling, FI, was determined with Eq. 2 [16]:

$$\text{FI} = \frac{\frac{\varepsilon_{\text{oligo}}}{\varepsilon_{F494}} \cdot A_{494}}{A_{260} - 0,35 \cdot A_{494}} \quad (2)$$

where $\varepsilon_{\text{oligo}}$ denotes the molar extinction coefficient of the ss or ds oligonucleotides. Absorption spectra were measured with a Cary 3E UV–VIS (Varian Inc., USA).

Table 1 Oligonucleotides sequences used in this study

Label	Sequence*
O1	5′-*FL*-CAA GGG TTC AAT GCA CTT GTC-3′
O2	5′-GAC AAG TGC ATT GAA CCC TTG-3′
O3	5′-*FL*-CAA GTT TTC AAT GCA CTT GTC-3′
O4	5′-GAC AAG TGC ATT GAA AAC TTG-3′
O5	5′-*FL*-CGC TTT GGA CAG TAC ACA GAT-3′
O6	5′-ATC TGT GTA CTG TCC AAA GCG-3′
O7	5′-CCT CAG GCG TA-3′
O8	3′-GGA GTC CGC A*F*A CCC AAG TT - 5′
O9	5′-*D* GGG TTC AAT GCA CTT GTC CAT-3′
O10	3′-ACGT GAA CAG GTA-5′

*Where *FL* denotes 5′-fluorescein, *D*—dabcyl-5′-dT and *F*—dT-fluorescein

Double stranded DNA preparations obtained after hybridization of ssDNA were purified on the HPLC ResourceQ column (Pharmacia Biotech., Sweden) equilibrated with 50 mM Tris buffer, 200 mM NaCl, pH 8.0 25 °C. The dsDNA samples were eluted with the NaCl gradient of 200–1,000 mM concentration. dsDNA were concentrated and desalted with the Centricon microconcentrators Ym-10 (cut-off 10,000 Da) (Amicon, USA).

The oligonucleotides used for molecular beacon formation

The oligonucleotides of the following sequences were used for the preparation of the molecular beacon:

D1:

5' – CCT CAG GCG TA – 3' O7
3' – GGA GTC CGC A**F**A **CCC AAG TT** – 5' O8

D2:

5' – **D GGG TTC AAT GCA CTT** GTC CAT – 3' O9
3' – **ACGT GAA** CAG GTA – 5' O10

O7 and O8 annealing produced a DNA half-site (D1) with a 9-bp overhang labeled with fluorescein (*F*)—fluorescence energy donor, whereas annealing O9 with O10 oligonucleotides produced a complementary DNA half-site (D2) labeled with dabcyl (*D*)—the acceptor of the fluorescence energy. Hybridization of D1 and D2 half-sites produced a full length DNA duplex (D1:D2) which contains the $hsp27_{\mathrm{pal}}$ sequence:

(D1:D2) which contains the $hsp27_{pal}$ sequence:

5' – CCT CAG GCG TA**D GGG TTC AAT GCA CTT** GTC CAT – 3'
3' – GGA GTC CGC A**F**A **CCC AAG TTA CGT GAA** CAG GTA – 5'

where *D* denotes dabcyl-5′-dT and *F*—dT-fluorescein. Absorption spectra of oligonucleotide solutions were recorded from 240 to 600 nm to determine the concentration of the samples and to control the quantity of labeling as described above. The DNA absorption coefficients used were ε_{260}=221,700 M^{-1} cm^{-1} (O7), ε_{260}=205,150 M^{-1} cm^{-1} (O8), ε_{260}=223,300 M^{-1} cm^{-1} (O9), ε_{260}=154,940 M^{-1} cm^{-1} (O10), all estimated from a weighted average of the mononucleotide extinction coefficients, according to the method described above.

Protein preparation

Protein preparations (UspDBD, $\mathrm{UspDBD}_{\Delta A}$, $\mathrm{UspDBD}_{\mathrm{E19}A}$ and $\mathrm{UspDBD}_{\mathrm{V71}A}$) were purified from an overproducing strain of *E. coli* as described previously [17, 18]. Before use, protein samples were centrifuged at 5,000 *g*, 4 °C for 15 min., and then concentrations were calculated using ε_{280}=7,000 M^{-1} cm^{-1} (UspDBD) according to [19].

Use of molecular beacons for detecting UspDBD protein: estimation of the dissociation constant

A molecular beacon assay has been recently described for detecting DNA binding proteins, which allows quantitatively analyzing the protein-dependent association of two DNA fragments, each containing about half of a DNA sequence-defining the protein binding site [4, 15].

The two-step experiment consists of equilibrium titrations of a donor-labeled DNA half-site with an acceptor-labeled DNA half site, and titrations of the donor- and acceptor-labeled DNA half-sites with the protein. First step data were analyzed by nonlinear regression fitting according to Eq. 3:

$$F = f_1 \cdot [D1] + f_2 \cdot [D1:D2] \tag{3}$$

where: F is the observed fluorescence intensity; f_1 — specific fluorescence intensity of D1; f_2—specific fluorescence intensity of full-length DNA duplex containing D1 and D2 ds oligonucleotides (D1:D2); [*D*1]—equilibrium concentration of the D1; [*D*1:*D*2]—equilibrium concentration of the D1:D2 duplex. Data points were analyzed according to Eq. 4:

$$F = F_{\mathrm{MAX}} + (F_{\mathrm{MIN}} - F_{\mathrm{MAX}}) \cdot \frac{(K_1 + [D1]_T + [D2]_T) - \sqrt{(K_1 + [D1]_T + [D2]_T)^2 - 4 \cdot [D1]_T \cdot [D2]_T}}{2 \cdot [D1]_T} \tag{4}$$

where F is the observed fluorescence intensity; F_{MAX}—maximal value of fluorescence intensity; F_{MIN}—fitted value of the minimal fluorescence intensity, K_1 is the dissociation constant of the D1:D2 complex; [*D*1], [*D*2]—equilibrium concentrations of the D1, D2, respectively; [*D*1:*D*2]—equilibrium concentrations of the [*D*1:*D*2] complex; $[D1]_T$, $[D2]_T$—total concentration of the D1, D2, respectively.

During the second step of the experiment, the equilibrium titration of both D1 and D2 with the protein, was performed. F is described now with Eq. 5:

$$F = f_1 \cdot [D1] + f_2 \cdot ([D1:D2] + [D1:D2:P]) \tag{5}$$

Experimental points were fitted to Eq. 6:

$$F = F_{\mathrm{MAX}} + (F_{\mathrm{MIN}} - F_{\mathrm{MAX}}) \cdot \frac{(K_{\mathrm{app}} + [D1]_T + [P]_t) - \sqrt{(K_{\mathrm{app}} + [D1]_T + [P]_T)^2 - 4 \cdot [D1]_T \cdot [P]_T}}{2 \cdot [D1]_T} \tag{6}$$

where, K_{app} is the apparent dissociation constant of the DNA-protein complex, $[P]_T$—total protein concentration. Taking into account the scheme of the protein (*P*) with

DNA (*D*1:*D*2) reaction and calculated values of K_1 and K_{ap}, one can obtain the K_d constant according to Eq. 7 [15]:

$$K_d = \left(K_{app}/K_1\right) \cdot D2 \tag{7}$$

Estimation of the apparent binding constants from steady-state fluorescence anisotropy measurements

Different amounts of the wild-type UspDBD protein or its mutants were incubated for 10 min. at 25 °C with the fluorescein-labeled DNA samples (25 nM) in 50 mM Tris–Cl, pH 7.8, 100 mM NaCl, 5 μM $ZnCl_2$, 1 mM 2-mercaptoethanol, 10% (v/v) glycerol. Dissociation constants were determined by plotting the change in fluorescence intensity at 518 nm (λ_{EX}=497 nm) against the total protein concentration. Equilibrium parameters of the data were computed by fitting the experimental points to the Eq. 8:

$$\Delta A = \Delta A_{max} \cdot \left\{ X - \left(X^2 - ([P]/(n[\mathrm{DNA}]))^{1/2}\right\}\right. \tag{8}$$

where

$$X = \frac{K_d + [P] + n[\mathrm{DNA}]}{2n[\mathrm{DNA}]},$$

$$\Delta A = A - A_F,$$

$$\Delta A_{max} = A_B - A_F,$$

A denotes the observed value of fluorescence anisotropy, A_F the fluorescence anisotropy of unbound DNA, A_B is the fluorescence anisotropy at saturation, [DNA] is the concentration of DNA, [P] is the protein concentration, K_d is the dissociation constant of the protein–DNA complex, and n is the number of equivalent non-interacting binding sites for UspDBD at the *hsp27*EcRE molecule. The changes of fluorescence anisotropy of the DNA titrated with proteins were corrected for two things: the dilution effect and the decrease of the time-dependent fluorescence signal of fluorescein-labeled $hsp27_{pal}$. The control measurement was performed in the same manner as described above, except the Tris buffer replaced the protein solution. All the parameters were obtained by non-linear least-squares analysis using the Origin program.

Results

Molecular beacon DNA probes provide a sensitive method for the quantitative detection of protein-induced DNA hybridization. To explore the specificity of the interaction on the level of point mutations it is indispensable to precisely determine the values of the association constants for the protein—DNA complex formation. Detection sensitivity was achieved through the application of the FRET methodology in molecular beacons.

Application of the molecular beacon for the K_d determination

Fluorescein and dabcyl are widely used fluorophore and quencher pair because dabcyl is non-fluorescent and can quench fluorescein extremely well. Dabcyl-modified oligonucleotides have broad and intense visible absorption in the range of 380–550 nm with maximum at around 480 nm (not shown) and directly superimposable with emission maximum of fluorescein (518 nm) making it useful as an acceptor in FRET application.

In the first step of the analysis, titration of the donor-labeled D1 half-site with a large excess of an acceptor-labeled D2 half-site in the absence of the protein was performed to obtain the K_1 dissociation constant for association of DNA half-sites with a 8-bp overhang (Fig. 2). The fluorescence emission spectrum of fluorescein-labeled D1 excited at 497 nm has its maximum at 518 nm. D1 treated with 0–400 nM of the dabcyl-labeled D2 half-site decreases the fluorescence intensity monitored at 518 nm by about 71%. The titration curve and the fit of the data points to the model described by Eq. 4 is shown in the inset to Fig. 2a. The calculated value of K_1 was 21.88± 2.86 nM.

Next, a mixture of the donor-labeled and acceptor-labeled half-sites was used to determine the dependence of fluorescence intensity on protein concentration (Fig. 2b). The titration curve and the fit of the data points to the model described by Eq. 6 is shown (inset to Fig. 2b).

The obtained value of K_{app} was 12.21±1.38 nM. Taking into account assumptions of the model (see Materials and methods) the dissociation constant of the protein-DNA complex (K_d) was calculated with Eq. 7 using K_{app} and K_1. Based on the constants K_d for the UspDBD–$hsp27_{pal}$ complex formation was calculated to be 1.42±0.28 nM (Table 2).

As shown with the electrophoretic mobility shift assay (EMSA) the mutated UspDBD preparation, in which 14 C-terminal amino acids from a region corresponding to *A*-box in RXR [20] were deleted ($UspDBD_{\Delta A}$), exhibited apparently the same binding pattern as the wild type UspDBD [17]. Interactions of $UspDBD_{\Delta A}$ with $hsp27_{pal}$ were analyzed in the same manner as the wild-type UspDBD. A mixture of D1 and D2 was used to determine the dependence of fluorescence intensity on $UspDBD_{\Delta A}$ protein

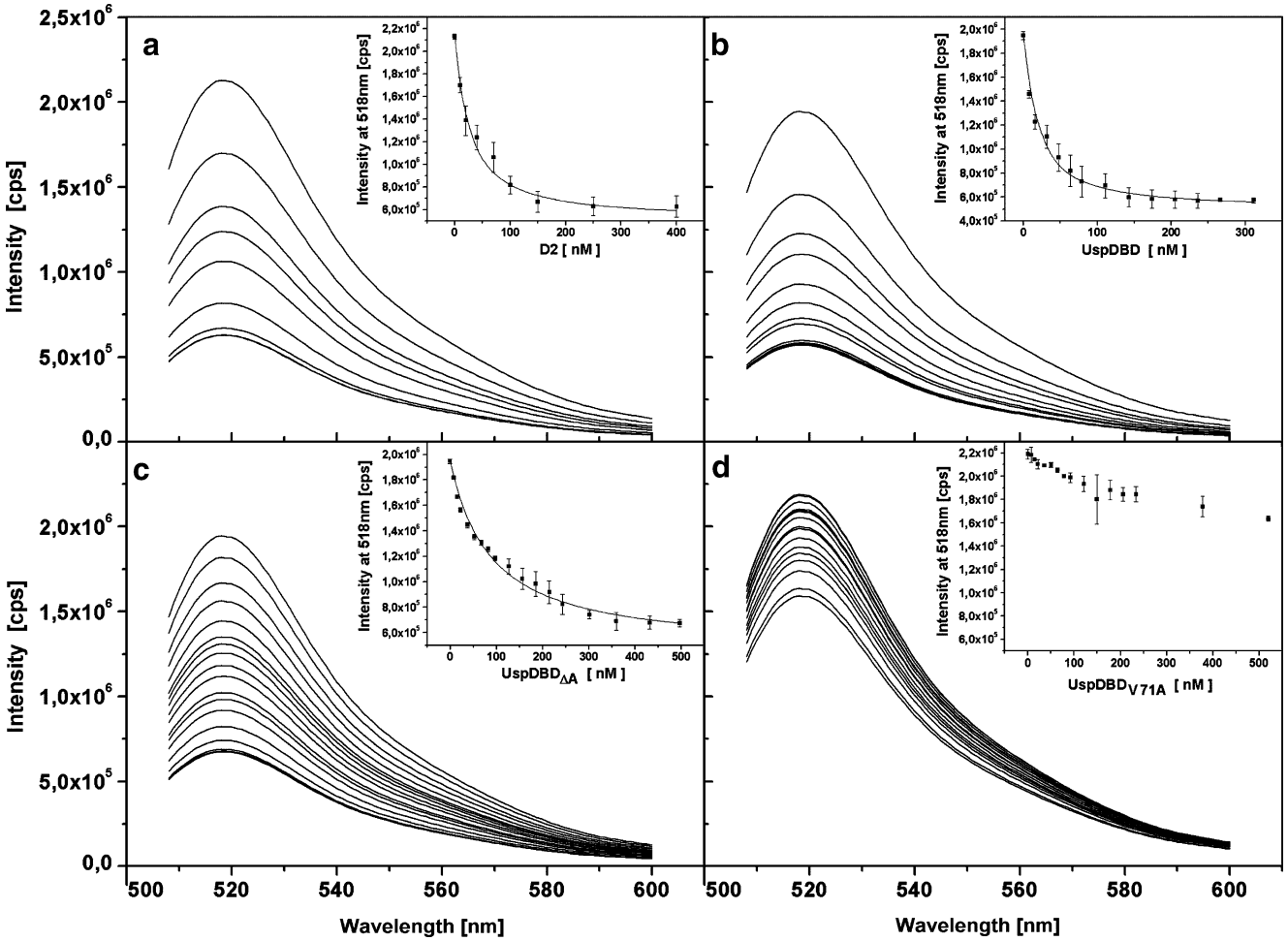

Fig. 2 Use of a molecular beacon for detecting the UspDBD–*hsp27*$_{pal}$ complex formation and estimation of the dissociation constant. **a** Association of fluorescence donor—(D1 labeled with fluorescein) 10 nM concentration and dabcyl-acceptor—(D2—dabcyl) labeled half-sites in the absence of the protein. D2 concentration dependence of the fluorescence signal. *Inset*: The titration curve of the donor-labeled DNA half-site with the acceptor-labeled DNA half-site (9 bp overhang) measured at 518 nm (λ_{EX}=497 nm). The *solid line* represents the fit of the data to Eq. 4 according to the model illustrated in Fig. 1. *Error bars* indicate standard deviation values of three measurements. **b** Titration of 10 nM donor-labeled (D1) and 12.5 nM acceptor—(D2) labeled half-site with the UspDBD protein. Inset: The titration curve measured at 518 nm (λ_{EX}=497 nm). The solid line represents the fit of the data to Eq. 6 according to the model illustrated in Fig. 1. *Error bars* indicate standard deviation values of three measurements. **c** Titration of 10 nM donor-labeled (D1) and 12.5 nM acceptor—(D2) labeled half-site with the UspDBD$_{\Delta A}$ protein. *Inset*: The titration curve measured at 518 nm (λ_{EX}=497 nm). The *solid line* represents the fit of the data to Eq. 6 according to the model illustrated in Fig. 1. *Error bars* indicate standard deviation values of three measurements. **d** Titration of 10 nM donor-labeled (D1) and 12.5 nM acceptor—(D2) labeled half-site with the UspDBD$_{V71A}$ protein. *Inset*: The titration curve measured at 518 nm (λ_{EX}=497 nm). The *solid line* represents the fit of the data to Eq. 6 according to the model illustrated in Fig. 1. *Error bars* indicate standard deviation values of three measurements. All the fluorescence spectra were obtained with excitation at 497 nm

concentration (Fig. 2c). The titration curve and the fit of the data points to the model described by Eq. 6 is shown (inset to Fig. 2c). The obtained value of K_{app} was 81.1±7.21 nM, whereas K_d=9.42±1.72 nM.

Analysis of the functional role of individual residues from the C-terminal fragment (the *T* box) of UspDBD has been previously presented [21]. The EMSA technique showed that the mutation of Val-71 with alanine significantly decreases affinity for *hsp27* and changes the overall protein structure as judged by CD spectroscopy [22]. Fig. 2d shows the changes of the fluorescence intensity of the D1 half-site in the presence of D2 which was titrated with UspDBD$_{V71A}$. Fluorescence intensity measured at 518 nm is quenched by about 25% in a wide range of the protein concentration (0–500 nM) (inset to Fig. 2d). It was not possible to reach saturation even at a high UspDBD$_{V71A}$ concentration, so a quantitative description of the binding was not meaningful. One can conclude that there is only weak protein–DNA interaction in comparison with the wild type UspDBD.

Table 2 Comparison of dissociation constants of the *hsp27*$_{pal}$ with UspDBD variants obtained with fluorescence anisotropy method and molecular beacon

Protein preparation	Fluorescence anisotropy (nM)	Molecular beacon (nM)
UspDBD	1.30±0,36	1.42±0.28
UspDBD$_{E19A}$	4.31±1,01	ND[1]
UspDBD$_{\Delta A}$	6.65±0.59	9.42±1.72
UspDBD$_{V71A}$	NB[2]	NB[2]

[1] Not determined, [2] Non-bonding mutant

Fluorescence anisotropy measurements of the UspDBD protein and protein mutants complex formation with *hsp27*$_{pal}$

Fluorescence anisotropy has been widely used to detect the formation of protein–DNA complexes [11, 13, 14, 16]. However, the changes of polarization are very low (typically a few hundredth parts of the anisotropy unit) and susceptible to the appearance of artifacts caused by light scattering. Binding UspDBD titrated with the natural hormone response element *hsp27*$_{pal}$ was followed by measuring the change in the steady-state fluorescence anisotropy of fluorescein-labeled DNA. Non-specific interactions were eliminated by poly(dIdC). An increase in the emission anisotropy (ca. 0.02 anisotropy unit) in the range of 1–100 nM protein concentration was observed (the relative change of anisotropy is presented in Fig. 3a). Calculation of the interaction parameters from Eq. 8 yielded K_d of 1.3±0.4 nM for the UspDBD–*hsp27*$_{pal}$ complex, the value close to that obtained with the molecular beacon technique. The number of independent, non-interacting binding sites was estimated to be 1.0±0.1.

To confirm the EMSA qualitative results and those obtained with the molecular beacon, fluorescently labeled *hsp*27$_{pal}$ was titrated with UspDBD$_{\Delta A}$ (Fig. 3b). Calculation of the interaction parameters from Eq. 8 yielded of 6.6±0.6 nM for the UspDBD$_{\Delta A}$–*hsp*27$_{pal}$ complex, a value comparable to that obtained with the molecular beacon.

Analogous experiments to those described above were done to determine the role of the *T*-box Val-71. Fluorescence anisotropy measurements (Fig. 3c), as well as molecular beacon analysis (Fig. 2c), demonstrated that Val-71 is essential for specific recognition of *hsp27*$_{pal}$ by UspDBD, since no interaction was observed between the response element and UspDBD$_{V71A}$.

It has been previously shown using EMSA experiments that in the nuclear receptor family Glu-19 from the *P*-box is an important residue for recognizing the regulatory element *hsp27* [23]. However, in the case of UspDBD the role of this residue is not clear, and is probably not engaged in interaction with *hsp27* [18]. In the present paper, to verify the role of the Glu-19 residue, fluorescence measurements were done for the protein mutant in which, Glu-19 was replaced with Ala (UspDBD$_{E19A}$) (Fig. 3d). The value of K_d=4.3±1.0 nM is comparable with the K_d obtained for the wild type UspDBD. Qualitative EMSA experiments gave a

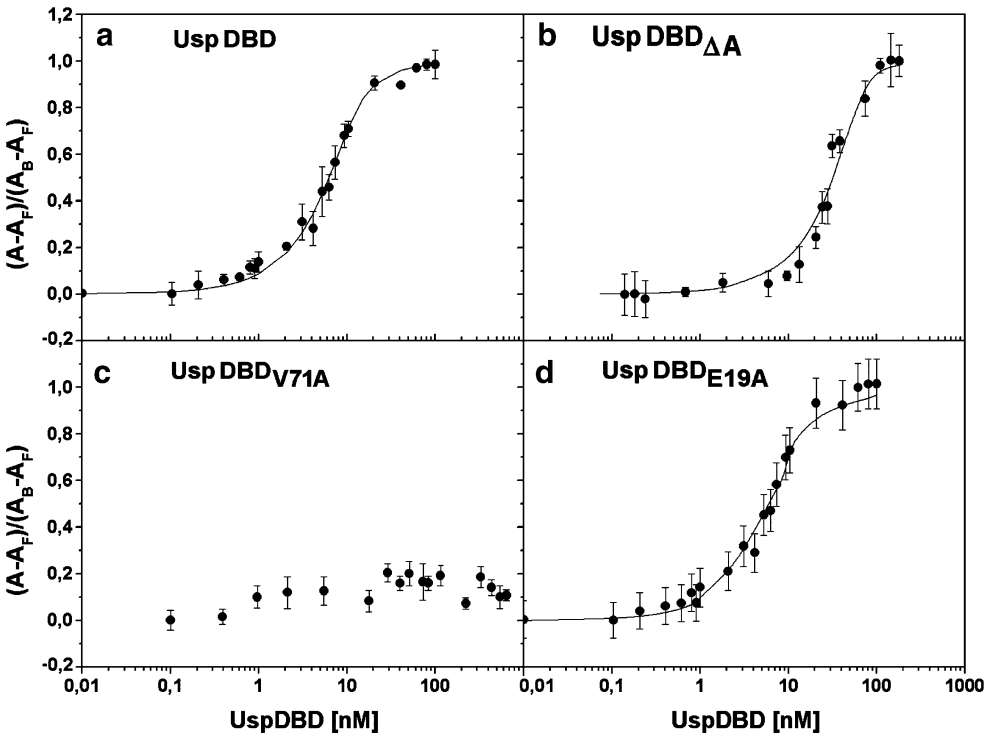

Fig. 3 Fluorescence anisotropy titrations of *hsp27*$_{pal}$ with different variants of UspDBD. **a** Titration of *hsp27*$_{pal}$ with the wild type UspDBD in 50 mM Na_2HPO_4, 100 mM NaCl, 10% glycerol, 5 μM $ZnCl_2$, 0.5 mg/mL BSA, 1 mM 2-mercapthoethanol, 10 nM poly(dIdC) pH 7.8 at 25 °C recorded at 518 nm (λ_{EX}=497 nm). The *solid line* represents the fit of the data to Eq. 8. *Error bars* indicate standard deviation values of three measurements. **b** Titration of *hsp27*$_{pal}$ with the UspDBD$_{\Delta A}$ mutant. All the conditions were the same as in Fig. 3a. **c** Titration of *hsp27*$_{pal}$ with the UspDBD$_{V71A}$ mutant. All the conditions were the same as in Fig. 3a. **d** Titration of *hsp27*$_{pal}$ with the UspDBD$_{E19A}$ mutant. All the conditions were the same as in Fig. 3a

value of K_d close to 50 nM for both proteins [18]. The difference could have arisen from the kinetic effects observed during electrophoresis especially at high protein concentration [24]. To sum up, we believe that Glu-19 is not important for the UspDBD–*hsp27* complex formation.

The aim of the control experiments was to verify the applicability of anisotropy measurements for the *hsp27*–UspDBD system. The single-stranded fluorescein-labeled DNA from $hsp27_{pal}$ and the non-specific dsDNA sequence of the same G-C content as a natural element were titrated with the UspDBD protein (Fig. 4a and b, respectively). A small increase of the emission anisotropy in Fig. 4a is observed probably due to non-specific interactions of the ss-oligonucleotide with UspDBD. No interaction of a random sequence with the UspDBD was observed. According to EMSA experiments [18] $hsp27_{pal}$ was mutated at the (−5, −6) positions, which seems to play a key role in the UspDBD–$hsp27_{pal}$ complex formation. The titration curve is presented in Fig. 4c. The small increase of the fluorescence anisotropy which appeared at high protein concentration (1 μM) testifies to the low protein affinity to the DNA sequence. The lack of saturation made K_d estimation impossible.

Discussion

Application of the molecular beacon methodology for the quantitative measurements of the $hsp27_{pal}$ and UspDBD interaction—comparison with the EMSA and fluorescence anisotropy measurements

The non-equilibrium EMSA technique has shown strong interaction of *hsp27* and UspDBD, however, K_d values were not determined [17, 18]. Recent experiments have demonstrated a poor fluorescence intensity change on the formation of a protein–DNA complex (data not shown) as well as a small change in the fluorescence anisotropy (ca. 0.02 anisotropy units). The molecular beacon is a considerable improvement over the approach in earlier studies and were successfully applied to obtain the K_d values. The present paper describes a quantitative analysis of the interaction of the DNA sequence from the natural regulatory element *hsp27*EcRE with UspDBD and the mutated element $hsp27_{pal}$ at the (−5T,−6T) position with the same protein. EMSA experiments showed a low affinity of this element to UspDBD [18]. We also analyzed mutated $UspDBD_{V19A}$ protein interaction with $hsp27_{pal}$. Glu-19 is known as one of three key residues for the specificity of the regulatory element recognition of the *P* cassette. All the nuclear receptor proteins have glutamate at the 19 position and demonstrate contacts of Glu-19 with DNA, particularly with *C* from G4–C4 bp and/or *A* from T3–A3 bp [25–29]. On the other hand, Grad *et al.* [18] indicated that Glu-19 is not indispensable for the regulatory sequence of $hsp27_{pal}$ recognition. This result is confirmed by the fluorescence anisotropy measurements (Table 2). The difference in K_d between the wild type and mutated protein was a few nM. The relationship between the K_d values obtained from fluorescence titration and qualitative EMSA results remains unchanged [18].

The data presented in the paper indicates that the molecular beacon methodology could be successfully used for the analysis of protein–DNA interaction. K_d value of the $hsp27_{pal}$–UspDBD complex measured with the molecular beacon (1.52±0.25 nM) is very close to the value obtained with the fluorescence anisotropy technique, 1.42±0.28 nM. Molecular beacons are preferable as a potential diagnostic

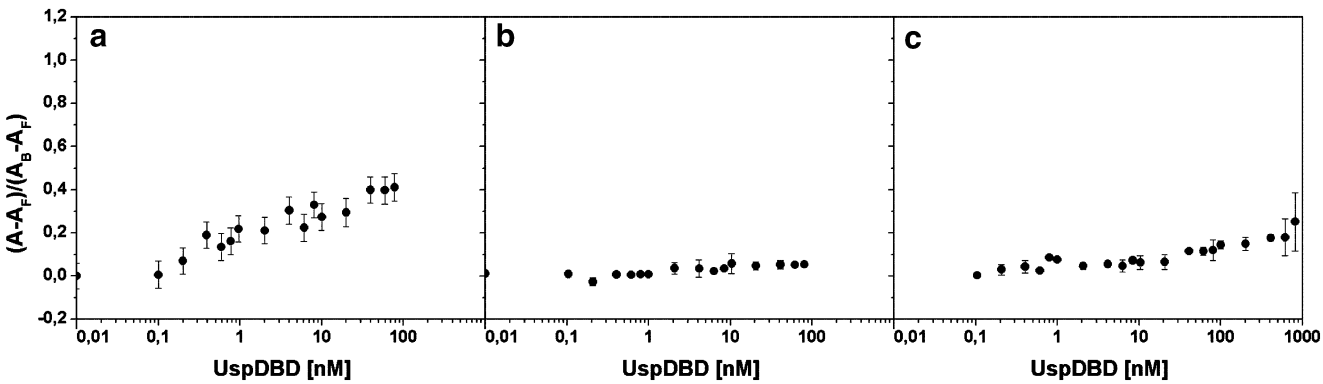

Fig. 4 Fluorescence anisotropy titrations of different variants of $hsp27_{pal}$ with UspDBD. **a** Titration of fluorescein-labeled ssDNA (O1) (10 nM) monitored at 518 nm (λ_{EX}=497 nm) as a function of the concentration of UspDBD in 50 mM Na_2HPO_4, 100 mM NaCl, 10% glycerol, 5 μM $ZnCl_2$, 0.5 mg/mL BSA, 1 mM 2-mercapthoethanol, 10 nM poly(dIdC), pH 7.8 at 25 °C. *Error bars* indicate standard deviation values of three measurements. **b** Titration of non-specific dsDNA sequence (10 nM) (O5–O6) with UspDBD. The DNA sequence contained the same number of GC bp as the wild type $hsp27_{pal}$. All the conditions were the same as in Fig. 4a. **c** Titration of $hsp27_{pal}$ (10 nM) mutated at −5 and −6 positions (O3–O4) with UspDBD. All the conditions were the same as in Fig. 4a

biosensor in which protein–DNA interaction plays a crucial role, because the strong fluorescence signal change on protein binding to DNA and the simple way to "bio-chip" formation [5, 30].

Fluorescence anisotropy measurements generate a rather large error because of small anisotropy changes caused by the unfavorable mass ratio of interacting molecules [11, 13, 14, 16].

It is interesting that the K_d values of the UspDBD and RXRDBD complexes with their regulatory elements differ significantly [27], despite the similarities of both proteins (DBDs' sequence identity equals 86%) [26].

It is worth noting that RXR exhibits less specific interaction with regulatory element DR1 in comparison with other receptor proteins such as TR and RevErb [26, 27].

The results presented above continue the investigation of the functional ecdysteroid receptor interaction with $hsp27_{pal}$. It seems that the molecular beacon methodology proposed for the first time by Heyduk, T. and Heyduk, E. [4] for the investigation of protein–DNA complexes, may be successfully applied to UspDBD–$hsp27_{pal}$. The advantage of the method is especially apparent when the fluorescence intensity or polarization signal is too low.

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