Kinetic analysis of Drosophila Vnd protein containing homeodomain with its target sequence

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Homeodomain (HD) is a highly conserved DNA-binding domain composed of helix-turn-helix motif. Drosophila Vnd (Ventral nervous system defective) containing HD acts as a regulator to either enhance or suppress gene expression upon binding to its target sequence. In this study, kinetic analysis of Vnd binding to DNA was performed. The result demonstrates that DNA-binding affinity of the recombinant protein containing HD and NK2-specific domain (NK2-SD) was higher than that of the full-length Vnd. To access whether phosphorylation sites within HD and NK2-SD affect the interaction of the protein with the target sequence, alanine substitutions were introduced. The result shows that S631A mutation within NK2-SD does not contribute significantly to the DNA-binding affinity. However, S571A and T600A mutations within HD showed lower affinity for DNA binding. In addition, DNA-binding analysis using embryonic nuclear protein also demonstrates that Vnd interacts with other nuclear proteins, suggesting the existence of Vnd as a complex. [BMB reports 2010; 43(6): 407-412]

INTRODUCTION

The homeodomain (HD), encoded by the 180-bp homeobox, is an evolutionarily conserved DNA-binding domain consisting of 60 amino acid residues. The proteins containing HD act as a regulator to specify positional information and segmental identity during development of all eukaryotes. Members of the HD family show a high degree of homology in their tertiary structures having three α-helices and a flexible arm in the N-terminal portion of the HD (1). The second and third helices of the HD form a helix-turn-helix DNA-binding motif. The third α-helix, also termed the DNA-recognition helix, and the flexible N-terminal arm are the primary regions which interact with DNA; the third α-helix binds in the major groove of the DNA and the flexible N-terminal arm is positioned in the adjacent minor groove (1). Although the highest degree of the amino acid sequence homology is found in the DNA recognition helix, there are differences in the amino acid sequences within this helix and the N-terminal region of the HD, which determines the DNA-binding specificity of the HD. Therefore, specific interaction of a protein containing the HD with its target DNA sequence is essential for its function to regulate the expression of the target gene.

Vnd (Ventral nervous system defective) is a crucial factor for neuroblast formation during the development of the embryonic central nervous system in Drosophila melanogaster (2). It is a member of the NK2 class containing HD (3). Like most NK2 class proteins, Vnd protein contains highly conserved regions, the HD and NK2-specific domain (NK2-SD). The NK2-SD consisting of 17 amino acids in length is located in the C-terminus of Vnd protein and is separated from the HD by a short linker. The NK2-SD might be involved in the regulation of gene expression by interacting with other transcription factors (4). Vnd HD contains the helix-turn-helix DNA-binding motif (5). The tertiary structure of Vnd HD is quite similar to those of the other members of the HD proteins, indicating the importance of the function of the HD proteins during evolution. However, the Vnd HD recognizes an unusual DNA consensus sequence 5'-T(T/C)AAGT(G/A)G-3', unlike many other HDs recognizing the canonical DNA consensus sequence containing 5'-TAATGG-3' in its core (6). In addition to the HD and NK2-SD at the C-terminal portion of the Vnd, there are a repression domain and three activation domains at the N-terminal region (7). Thus, Vnd is a dual regulator functioning as both a repressor and an activator in regulating the expression of target genes (8).

The interaction of Vnd with its target sequence has been studied by using nuclear magnetic resonance spectroscopy and electrophoretic mobility shift assay (9, 10). Although these techniques have been successfully employed for the analysis of binding specificity and affinity between transcription factors and their targets, they have limitations in studying the dynamics of the DNA-protein interaction. Recently, optical biosensors based on the phenomenon of surface plasmon resonance (SPR) have been used to determine the specificities, affinities, and kinetics of biomolecular interactions such as protein-protein, protein-nucleic acid, and even eukaryotic cell in-
Kinetic analysis of Vnd with its target sequence
Suik Yoo

interactions (11, 12). Optical biosensors provide several advantages that they are sensitive enough to detect small changes in molecules, do not require high protein concentration, monitor interactions in real-time, and enable the label-free detection of the biomolecules.

In this study, SPR technology was employed to determine the binding characteristics of the Vnd to its target DNA. The recombinant proteins containing the full-length Vnd (Vnd-FL) and HD/NK2-SD region of Vnd (Vnd-HN) were expressed, and the kinetic analysis of the proteins to target sequence was performed using Biacore 3000. In addition, the effects of mutations within either HD or NK2-SD on binding affinity were analyzed.

RESULTS AND DISCUSSION

Binding affinity of the Vnd recombinant protein containing either full-length or homeodomain/NK2-specific domain to the target sequence

To determine the binding affinity of Vnd protein to its target DNA, recombinant proteins containing either the full-length Vnd (termed Vnd-FL: amino acid residues 1-723) or HD/NK2-SD (termed Vnd-HN: amino acid residues 537-650) carrying a histidine-tag at the N-terminus were overexpressed in E.coli and purified by affinity chromatography using nickel-chelating column (Fig. 1). The consensus oligonucleotide (ligand) for Vnd binding was biotinylated, and immobilized on the sensor chip SA via biotin-avidin interaction. To offset the non-specific binding of the protein on the surface of sensor chip, a Twist target sequence was used as a control. Twist is a transcription factor containing helix-loop-helix motif, and recognizes 5’-CATATG-3’ core sequence (13), which is quite different from the Vnd core motif, 5’-TAAGTG-3’. After passing the recombinant protein (analyte) over the target sequence on the sensor chip, the amount of bound molecules was detected by SPR and displayed as response units (RU) versus time in a sensorgram.

For kinetic analysis, association of the protein-DNA was performed for 2 min followed by a 5-min dissociation to measure the association constant, $k_a$ and dissociation constant, $k_d$ respectively (14). The binding affinity, $K_D$ was calculated as $k_d/k_a$. Since the molecular weight of Vnd-FL is approximately 5-times higher than that of Vnd-HN, the sensorgrams show higher RU for Vnd-FL (Fig. 2A) compared to the one for Vnd-HN (Fig. 2B), representing linear correlation between MW and RU.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Schematic representation of the domains of the Vnd protein. (A) Full-length Vnd containing 723 amino acids flanked by histidine-tag at the N-terminus (Vnd-FL). The numbers above the line indicate amino acid residue: RD, repression domain (amino acid residues 1-193); Q, glutamine-rich domain (293-334); AcD, acidic domain (401-458 and 509-528). The activation domains of the Vnd consist of Q and two AcD. (B) C-terminal region of vnd containing homeobox and NK2-SD (Vnd-HN). The amino acid residues from 537 through 650 are shown at the bottom. HD and NK2-SD are underlined, and three helices within HD are shown by bracket above the sequence. The amino acids substituted by site-directed mutagenesis are indicated by black-filled box.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** Kinetic analysis of the Vnd binding to its target sequence. (A) Sensorgrams showing interaction of Vnd-FL with the target DNA. Sensorgrams shown by colored lines indicate different protein concentrations serially diluted by 2-fold from 100 nM (top) to 6.25 nM (bottom). The black lines represent a global fit of each data set to 1 : 1 binding by mass transport. The arrow indicates end point of injection. (B) Sensorgrams showing interaction of Vnd-HN with the target DNA. Colored lines represent protein concentrations from 100 nM (top) to 3.125 nM (bottom). Each sensorgram was overlaid and zeroed on the y-axis to the start injection time, and on the x-axis to the average baseline.
The $K_0$ value of the Vnd-FL was first determined in this study, and the Vnd-HN was shown to bind to its target DNA with nanomolar affinity (Table 1), consistent with the previous study (15). The $K_0$ value shows that the binding affinity of Vnd-HN is 34 times higher than that of Vnd-FL (Table 1). Although the binding affinity of Vnd-FL is decreased at a moderate level, the association and dissociation rates are strongly reduced by 5-6 orders of magnitude. Since Vnd contains many domains, it might be possible that DNA-binding domain is not fully exposed in the absence of cofactors.

**Kinetic analysis of alanine substitutions within either homeodomain or NK2-specific domain**

The ability of HD protein to bind its target is strongly dependent on the specific amino acid residues in the HD. Previously, it has been reported that the amino acid residues at the positions 3 and 5 (Vnd 547 and 549, respectively) of the N-terminal arm as well as residues at the positions 47, 50, and 34 (Vnd 591, 595, and 598, respectively) of the recognition helix in HD directly contact with the bases of the DNA (5, 9, 16). In addition to the residues directly involved in base-specific interactions, amino acid side chains at the positions that do not contact any DNA bases are believed to affect the interaction of the HD with its target DNA, and thus may be important functionally. Vnd can be extensively phosphorylated in vivo (17). Since the phosphorylation of a protein greatly influences its function generally, mutations at the phosphorylation sites of the Vnd might affect DNA binding. To test this possibility, the putative phosphorylation sites within HD were replaced with alanine. The S571A and T600A mutant proteins were subjected to kinetic analysis (supplementary Fig. 1A and 1B). The binding affinity of S571A and T600A proteins was decreased at moderate level, approximately 23- and 2-fold, respectively, compared to that of Vnd-HN (Table 1). Although the binding affinity of Vnd-FL is decreased at a moderate level, the modest decrease in binding affinity of the alanine mutants is due to the combination of slow association and slow dissociation (Table 1).

NK2-SD composed of a conserved 17 amino acid sequence is present at the C-terminus of the NK2-type proteins (Fig. 1B). NK2-SD contributes to the repression activity of Vnd by stabilizing the interaction of Vnd with Groucho (18), thus functioning as an accessory DNA-binding domain. To determine whether NK2-SD is involved in the DNA binding, a putative phosphorylated residue was replaced with alanine (S631A), and the kinetic analysis was carried out. The result showed that the association/dissociation rate constants and equilibrium dissociation are almost same as Vnd-HN (supplementary Fig. 1C and Table 1), suggesting that the serine residue at the 631 position is not involved in the DNA binding. This is good agreement with the previous report that NK2-SD does not contribute significantly to either the specificity or the affinity of DNA binding (19).

**Interaction of Vnd with other proteins in nucleus**

To understand the precise mechanism of a transcription factor on the regulation of its target gene, it is necessary to identify its binding partner such as cofactors. One of the advantages using Biacore system is the ability to recover the bound materials from the surface by analyte recovery, called ligand fishing (20, 21). In a typical experiment, a specific ligand (Vnd target sequence) is first immobilized on the carboxymethyl-dextran surface of the sensor chip. Then, analyte (nuclear proteins) solution is passed over the sensor chip, and binding of analyte molecules to the ligand is recovered. In this study, to increase the efficiency of recovery, all four flow cells were used. First, neutravidin was immobilized on the sensor chip CM5 by amine coupling instead of using the sensor chip SA since neutravidin shows the highest specificity for biotin and lowest nonspecific background (22). The maximum level of neutravidin bound to each chip surface was approximately 15 kRU (bidirectional arrow in Fig. 3A), corresponding to 15 ng. The sensogram of analyte recovery was shown in Fig. 3B. After passing the nuclear soluble proteins over the target sequence attached to the sensor chip via neutravidin-biotin interaction, the bound molecules was eluted by injecting 0.05% SDS at 1500 RU (bidirectional arrow in Fig. 3B).

For analyte recovery experiment, bacterial soluble proteins

<table>
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<th>Figure</th>
<th>Analyte</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (M$^{-1}$)</th>
<th>Fold loss compared to Vnd-HN$^{a}$</th>
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<td>Suppl. 1C</td>
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<td>$3.49 \times 10^{11}$</td>
<td>$1.58 \times 10^4$</td>
<td>$4.52 \times 10^{10}$</td>
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$^a$K$_0$ value (affinity) was calculated by $k_a/k_d$.$^b$The relative changes in association/dissociation constants and affinity compared to Vnd-HN are shown for the Vnd-FL and mutants.

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extracted from pET-HN bearing bacteria were first applied, and the recovered molecules were analyzed by SDS-PAGE. For comparison, the Vnd-HN protein purified from Ni-chelating column chromatography was also analyzed by SDS-PAGE as well. The result demonstrates that MW of the recovered protein, R1, is exactly the same as the one of the Vnd-HN protein indicated by arrowhead (Fig. 3C) representing the efficacy of Biacore system. Next, the nuclear pellet/soluble proteins were applied to the system, and the recovered molecules were shown by silver staining. Consistent with the previous data (17, 18), several bands (arrows in Fig. 3D) from the nuclear soluble fraction (R3) were detected including the endogenous Vnd (arrowhead in Fig. 3D), strongly supporting that Vnd interacts with other cofactors in nucleus. In contrast, there is no protein band detected in the nuclear pellet fraction (R2). Currently, we are carrying out experiments to identify the unknown bound molecules to Vnd.

In conclusion, the binding affinity of the Vnd-mutant proteins carrying an alanine substitution at the putative phosphorylation sites within HD or NK2-SD was compared with Vnd-HN. In contrast to the moderately reduced DNA-binding affinity of the mutant proteins (S571A and T600A), the association and dissociation rates were dramatically decreased, resulting in both slow binding and slow dissociation. However, the mutation within NK2-SD (S631A) did not influence the DNA-binding affinity, suggesting that NK2-SD is not involved in the interaction of the Vnd with its target DNA. In addition, the interaction of Vnd with other proteins was successfully detected using SPR. This is the first demonstration of kinetic study of Vnd transcription factor.

MATERIALS AND METHODS

Plasmid construction

Full-length vnd DNA fragment corresponding to amino acid 1-723 was amplified by polymerase chain reaction (PCR) using vnd cDNA clone as a template and primers, FL-F and FL-R. The PCR product was digested with EcoRI and SalI, and the resulting fragment was cloned into EcoRI- and SalI-digested pET30b bacterial expression vector to generate pET-FL. To generate pET-HN expressing HD and NK2-SD region from Vnd protein, DNA fragment corresponding to amino acid 537-650 was amplified by PCR using HN-F and HN-R primers. After digestion with EcoRI and SalI primers, the fragment was cloned into pET30b vector.

For alanine substitutions, each point mutation within the HD/NK2-SD region was introduced by following QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer’s protocol (Stratagene). Briefly, an inverse PCR was carried out using pET-HN as a template and overlapping primer sets, S571A-F/S571A-R, T600A-F/T600A-R, and S631A-F/S631A-R for S571A, T600A, and S631A mutation, respectively. The resulting PCR reaction was treated with DpnI to digest the parental DNA, and was subjected to transformation. The vnd sequences in each construct were confirmed by DNA sequencing. The primer sequences were shown in the supplementary Table 1.
Purification of recombinant proteins

Expression of the recombinant Vnd proteins in E. coli was carried out by following pET expression system (Novagen). Briefly, plasmid DNA was transformed into BL21 (DE3), and protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After incubation for 3 h, the cells were collected and the proteins were purified by nickel-chelating column chromatography. The concentration and purity of the protein was determined by spectrophotometer and SDS-PAGE, respectively.

Kinetic analysis

All experiments were carried out at 25°C using Biacore 3000 SPR sensor (Biacore) and sensor chip SA carrying a dextran matrix to which streptavidin has been covalently attached. The sensor chip SA was washed by three consecutive injections with 1 M NaCl in 50 mM NaOH for 2 min at a flow rate of 50 μl/min to remove any un conjugated streptavidin. For preparation of Vnd target sequence, biotinylated sense oligonucleotide (5'-CGGTAYGTTAAGTTGGCCG, core binding site is underlined) was mixed with anti-sense oligonucleotide (5'-GCCAACCCTAATCTAAGCCG) at equal molar concentration. The mixture of sense and antisense oligonucleotide was heated at 95°C for 10 min, and slowly cooled down to room temperature to generate double stranded DNA. To immobilize the Vnd target DNA on the surface of sensor chip, 5 μM of DNA in 800 mM NaCl was injected manually at a low flow rate (5 μl/min) over a single flow cell until a resonance unit (RU) value reaches 100-200. Twist target sequence (sense, flow rate (5 μm) mobilize the Vnd target DNA on the surface of sensor chip, 1 μM in 50% bleach solution. The embryos were homogenized in 10 embryo volumes of buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 250 mM sucrose, 1 mM PMSF), filtered through 120 μm nylon mesh, and then centrifuged at 1,000 g for 10 min. The pellet was washed twice in 5 embryo volumes of buffer A by centrifugation, and resuspended in 1 embryo volume of buffer B (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2). The resuspended purified nuclear pellet was incubated at 37°C for 15 min in the presence of 8 μg RNaseA and 10 μg DNaseI, and then centrifuged at 1,000 g for 10 min. The supernatant was collected for the nuclear soluble fraction. The pellet was resuspended in 0.45 embryo volume of buffer C, 0.05 embryo volume of 1 M Tris-HCl, pH 7.5, and 0.5 embryo volume of 2 M NaCl to dissolve nuclear pellet proteins. After incubation on ice for 10 min, the suspension was centrifuged at 10,000 g for 30 min, and the supernatant was harvested for nuclear pellet fraction. Both nuclear soluble and pellet fractions were dialyzed in HBS-EP running buffer using Slide-A-Lyzer® Dialysis Cassette (Pierce).

Analyte recovery

Neutradin (Pierce) was immobilized on the surface of sensor chip CM5 carrying a dextran matrix following a standard amine coupling protocol (Biacore). The chip surface was first activated by injection of 0.4 M EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] and 0.1 M NHS (N-hydroxysuccinimide) at a flow rate of 5 μl/min for 7 min. Neutradin was diluted to 50 μg/ml in 10 mM acetate pH 4.5, and injected at 5 μl/min for 10 min followed by a 7 min injection of 1 M ethanolamine, pH 8.5 to inactivate the residual active groups. After immobilization of neutradin, Vnd target sequence was injected at 5 μl/min to reach the maximum level. All four flow cells were used for analyte capture and recovery following Analyte Recovery wizard of Biacore 3000. The protein sample was injected at 5 μl/min for 5 min, and then the surface of sensor chip was washed with HBS-EP buffer. The bound protein was eluted with 2 μl of 0.05% SDS, and analyzed by SDS-PAGE followed by staining using either NOVEX® Colloidal Blue Staining Kit (Invitrogen) or SilverQuest™ Silver Staining Kit (Invitrogen).

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