

# Surface Plasmon Resonance Imaging of Transcription Factor Proteins: Interactions of Bacterial Response Regulators with DNA Arrays on Gold Films<sup>†</sup>

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Received February 6, 2002. In Final Form: April 19, 2002

Surface plasmon resonance (SPR) imaging measurements have been used to study the sequence specific adsorption of response regulator proteins to DNA arrays constructed on gold thin films. Bacteria adapt to their environment primarily through two-component signal transduction systems that consist of an environmental sensor histidine kinase and a transcription activator response regulator DNA binding protein. DNA arrays were fabricated from oligonucleotide sequences of known DNA binding regions for two response regulators: OmpR, which controls gene expression of the outer membrane porin proteins in *Escherichia coli*, and VanR, which is involved in the antibiotic vancomycin resistance in *Enterococcus faecium*. The label-free method of SPR imaging was then used to monitor the sequence specific binding of these two response regulator proteins to the DNA arrays. The promoter regions from the *ompF* (F1 and F2) and *ompC* (C1) genes for the OmpR protein, and from the *vanRS* (R1) and *vanHAX* (H1 and H2) genes for the VanR protein were studied. SPR imaging was used to (i) monitor and compare the binding of both response regulators to various promoter regions on the DNA array, (ii) compare the binding of the OmpR response regulator protein in its phosphorylated and nonphosphorylated forms, and (iii) monitor the inhibition of VanR protein binding to the DNA arrays in the presence of a small molecule DNA binding inhibitor. The proteins exhibited specificity for the known binding sequences compared to control sequences on the DNA array. For the OmpR protein, the highest amount of binding was observed at the F1 site. When OmpR was phosphorylated by a small molecule phosphodonator, acetyl phosphate, there was on average a 42% increase in protein binding. In contrast, phosphorylated VanR binding decreased by an average of 40% in the presence of a known DNA binding inhibitor, (2,3,4-trifluorophenylisothiazolone). These measurements demonstrate that SPR imaging is an effective screening method for compounds that target DNA protein interactions and can serve as a useful tool for the discovery of new therapeutic molecules that target DNA binding proteins.

## Introduction

The interactions between transcription regulatory proteins and their DNA binding sites are key aspects in the control of gene expression and the regulation of genetic information. The general structural motif that is found in the DNA binding region of many transcription regulatory proteins, the helix–turn–helix structure, can be altered to recognize different target sequences through variations in the amino acid side chains or the linker that connects the structural domains.<sup>1–3</sup> Unlike DNA–DNA hybridization interactions responsible for the genetic code, the interactions between an amino acid and a nucleic acid base pair are not a one to one relationship.<sup>4</sup> Therefore, the DNA region that a particular transcription factor binds to cannot necessarily be deduced from the protein's amino acid sequence. In addition, many of these proteins bind

to a collection of similar DNA sequences, which can exist in the promoter region of one or more genes. It is this sequence-specific binding of the transcription factors that underlies their role in the control of gene expression. Traditional methods that have been used to analyze DNA–protein interactions include gel shift analysis,<sup>5</sup> DNA footprinting,<sup>6</sup> and fluorescence polarization.<sup>7</sup> These methods provide reliable results but do not lend themselves to multiplexed studies of DNA–protein interactions.

Surface plasmon resonance (SPR) imaging is a surface-sensitive spectroscopic technique that can be used to simultaneously monitor the interactions of many biomolecules immobilized on a thin gold film without the use of fluorescent, enzymatic, or radioactive tags. SPR imaging has been used previously to monitor DNA–DNA hybridization, RNA–DNA hybridization, and DNA–protein interactions on surfaces.<sup>8–11</sup> For example, we have recently

<sup>†</sup> Part of the *Langmuir* special issue entitled The Biomolecular Interface.

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shown that SPR imaging experiments can be used to monitor the interactions of single-stranded binding protein and the mismatch binding protein, MutS, to surface-bound DNA arrays.<sup>10,11</sup> Studying sequence-specific DNA–protein interactions in an array format with SPR imaging provides the advantage of examining the interactions of a particular protein with many DNA sequences (possibly the entire genome) in one step. DNA arrays are easily constructed and can be designed to include sequences from the promoter regions of one or many genes, or they can be constructed from a combinatorial mixture of nucleotides. In addition, SPR imaging measurements with DNA arrays offer the possibility of directly studying proteins from a sample without the need for protein modification (i.e., fluorescent tagging) or prior knowledge of the protein's structure or function.

In this paper, we report the use of DNA arrays and SPR imaging to study the sequence-specific binding of transcription regulatory proteins known as response regulators. Response regulators are part of bacterial two-component signal transduction systems, the primary means by which bacteria sense and adapt to environmental changes.<sup>12–14</sup> These systems consist of two conserved components, a sensor histidine kinase protein that auto-phosphorylates in the presence of an appropriate environmental stimulus and a response regulator protein. Both response regulator and sensor kinase proteins are phosphoproteins that can exist in an “active” (phosphorylated) and an “inactive” (unphosphorylated) form. A phosphorylated sensor kinase can convert a response regulator from its inactive form to its active form, which has an increased affinity for a particular DNA binding site. In bacteria, these two-component systems typically control transcriptional activation of genes by binding upstream or inside of a gene's promoter.<sup>15</sup> Promoter regions of approximately 12 nucleotides have been identified as sites of response regulator binding using DNA footprinting studies.<sup>6</sup>

The exact nature of the response regulator's involvement in signal transduction is still unknown for many two-component systems.<sup>16</sup> Since these systems have been found to be involved in antibiotic susceptibility, tolerance, and resistance in pathogenic bacteria, there is considerable interest in mapping these networks to understand their role in these problematic health issues.<sup>17</sup> There is generally a one to one association between a histidine kinase and a response regulator, and this relationship can often be inferred on the basis of the location of these genes within the bacterial genome. However, the promoter site with which the response regulator controls gene expression cannot be deduced in this fashion as the controlled genes do not necessarily associate with the regulatory protein genes and may in fact be quite distantly located.

In principle, SPR imaging can be used to determine regions in a bacterial genome where a response regulator binds and thus identify the genes regulated by a particular two-component system. To demonstrate the utility of SPR imaging in the detection of response regulator binding, we constructed arrays of oligonucleotides composed of

known DNA binding regions for two response regulator proteins: OmpR, a well-studied response regulator that controls the synthesis of the outer membrane porin proteins,<sup>15</sup> and VanR, a response regulator involved in the antibiotic vancomycin resistance.<sup>17</sup> SPR imaging was then used to monitor the binding of OmpR and VanR proteins to these DNA arrays. The amount of protein binding to the various promoter segments was quantitated and compared. We also monitored and compared the binding of the response regulators to these DNA arrays in their phosphorylated (OmpR-P or VanR-P) and non-phosphorylated (OmpR or VanR) forms. Finally, we studied the binding of the VanR protein in the presence of a known DNA binding inhibitor, CpdA, to show that this method is also feasible for screening potential DNA binding inhibitors of response regulator proteins. This last measurement demonstrates that SPR imaging may be useful in the discovery of compounds that disrupt the formation of response regulator–promoter DNA complexes and alter gene expression in bacteria.

## Experimental Considerations

**Materials.** Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC, Pierce), 11-mercaptoundecylamine (MUAM, Dojindo Laboratories), 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-NHS, Novabiochem), *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid MW 2000 (PEG-NHS, Shearwater Polymers), acetyl phosphate lithium potassium salt, HEPES, magnesium chloride, and urea (Sigma), sodium chloride (Aldrich), disodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate monohydrate (Fluka), and 2,3,4-trifluorophenylisothiazolone (CpdA, Maybridge Chemical Co.) were used as received. All rinsing steps were performed with absolute ethanol and Millipore-filtered water. The 5' thiol modifier C6 (Glen Research) DNA sequences with a 15 base Thymine spacer (see Table 1) were synthesized at the University of Wisconsin Biotechnology Center on an ABI DNA synthesizer and were deprotected prior to use as outlined by Glen Research Corp.<sup>18</sup> Complementary sequences were synthesized as above but contained no modifications or spacers. Before use, each oligonucleotide was purified using reverse-phase binary elution HPLC (Shimadzu SCL10AVP) and the concentration was determined using a HP8452A UV–vis spectrophotometer.

**Response Regulator Protein Isolation and Phosphorylation.** The VanR response regulator protein was expressed as a GST fusion protein using the complete VanR sequence and the plasmid pGEX-2TK (Amersham Pharmacia Biotech) in *E. coli* BL21  $\lambda$  DE3 cells as previously reported.<sup>19</sup> OmpR protein was expressed as a glutathione S-transferase (GST) fusion protein in a similar fashion using the complete OmpR sequence. Response regulator phosphorylation was performed as previously described,<sup>19,20</sup> with a total reaction volume of 100  $\mu$ L containing 50 mM HEPES (pH 7.2), 5 mM MgCl<sub>2</sub>, 9  $\mu$ M response regulator, and 50 mM acetyl phosphate. This mixture was incubated at 37 °C for 1 h and was used immediately for SPR imaging experiments or stored on ice prior to use. The amount of protein phosphorylated by a small molecule phosphodonator, such as acetyl phosphate, has been reported to range from 60 to 90% of the total protein, depending on the reaction time and conditions.<sup>21</sup> Separation of the phosphorylated protein from the nonphosphorylated protein can be attained by purifying the mixture using reverse-phase HPLC, as has been demonstrated by others.<sup>22</sup> All studies reported here involving phosphorylated response regulator contained both

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the phosphorylated protein and a small amount of nonphosphorylated protein.

**DNA Array Fabrication.** Gold films (45 nm) with a thin chromium underlayer (1 nm) were vapor deposited on SF10 glass slides (Schott Glass Technologies) in a Denton Vacuum DV-502A evaporator, and used for all SPR imaging measurements. The multistep procedure used to create DNA arrays on gold films involving a combination of self-assembly and UV photopatterning has been previously reported.<sup>10</sup> Briefly, a bare gold surface was modified with a self-assembled monolayer of the amine-terminated alkanethiol MUAM. The amine-terminated surface was then modified with the hydrophobic protecting group Fmoc. Using a quartz mask with 500  $\mu\text{m} \times 500 \mu\text{m}$  square features, the surface was UV photopatterned with a mercury xenon arc lamp for 1 h at 400 W. The sample was then immersed into the MUAM solution to react with the bare Au patches that remained after photopatterning. The hydrophilic wells, surrounded by a hydrophobic background of Fmoc, were then reacted with a bifunctional linker SSMCC, followed by thiol-modified DNA. Small volumes of these solutions (40 nL or less) were delivered to the surface using a PV830 Pneumatic Pico Pump (World Precision Instruments). After immobilization of DNA, the Fmoc background was removed in a mild base and reacted with PEG-NHS to form a PEG-coated background layer to prevent the nonspecific adsorption of protein to the array background. After each reaction step, the array was rinsed with water and/or ethanol and dried in a stream of nitrogen. Following modification with PEG, the array was assembled into a sample cell assembly and used immediately for SPR imaging experiments.

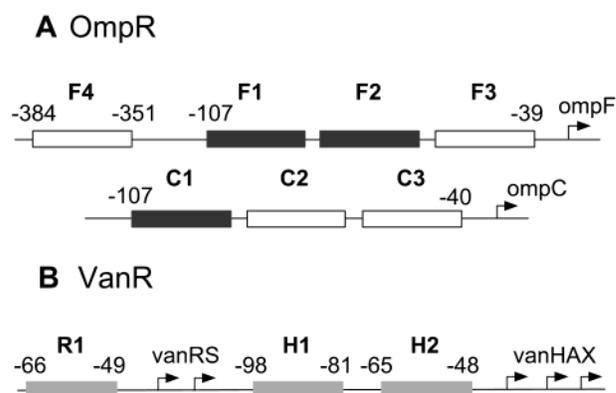
**Surface Plasmon Resonance Imaging.** The in situ SPR imaging apparatus has been reported elsewhere.<sup>10,23</sup> Briefly, p-polarized collimated white light was directed toward a prism/Au thin film/buffer assembly at a fixed angle. Light reflected from this assembly was passed through a narrow band-pass filter ( $\lambda = 800 \text{ nm}$ ) and collected by a CCD camera (GWC Instruments). Prior to exposing the array to a solution of the response regulator protein, the single-stranded DNA (ssDNA) array was exposed to DNA sequences complementary to the immobilized oligonucleotides to create a double-stranded DNA (dsDNA) array. A solution of the response regulator protein in phosphate buffer (pH 7.1), 5 mM  $\text{MgCl}_2$ , and 100 mM NaCl with a total volume of 350  $\mu\text{L}$  was flowed into the sample cell holding the array using a peristaltic pump (Instech Laboratories). All SPR images were taken at equilibrium conditions, 15 min after introducing protein into the flow cell. An image taken prior to exposing the array to the protein solution was subtracted from this image to obtain the SPR difference images shown in Figure 2. The protein and DNA complement were removed from the surface using an 8 M urea solution. The arrays were used for approximately 20 cycles of DNA hybridization and protein binding experiments before degradation of the array was observed, as determined by a decrease in the SPR signal for hybridization of complementary DNA. For the VanR inhibition experiments, 5  $\mu\text{M}$  CpdA was added to the diluted protein solution and left to react for 15 min prior to introducing the sample into the flow cell containing the DNA array.

**Data Analysis.** All SPR Images were collected using the software program XCAP v1.0 (EPIX Inc.) and further analyzed using the NIH Image v.1.61 software package. The line profile option from this software was used to collect the data reported in Figures 3–6. The numerical values reported in Figures 4–6 were collected by integrating the area under the plot profile and then normalizing this value to account for the amount of double-stranded DNA immobilized on the array. This was inferred from the SPR signal obtained upon hybridizing DNA complements to each immobilized sequence.

## Results and Discussion

**A. The OmpR and VanR Response Regulator Promoter Regions.** We chose to study the OmpR response regulator binding because it is a well-characterized protein and its target promoter regions are known in

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**Figure 1.** Schematic presentations. (a) The regulatory region of the *ompF* and *ompC* genes consisting of F1, F2, F3, F4 or C1, C2, C3 OmpR binding sites, respectively. The numbers by the OmpR binding sites represent the base locations relative to the transcriptional start site. (b) The regulatory region of the *vanRS* and *vanHAX* genes consisting of the R1 and H1, H2 binding sites, respectively. The numbers next to the binding regions are relative to the *vanR* and *vanH* transcriptional start sites. The shaded regions represent those used in this study (see Table 1 for a list of sequences).

detail.<sup>15,22,24,25</sup> In *E. coli*, OmpR and its cognate sensor kinase, EnvZ, control the expression of the outer membrane porin proteins, OmpC and OmpF. In response to changes in environmental osmotic pressure, OmpR binds in a hierarchical fashion to the promoter regions of the *ompF* and *ompC* genes.<sup>15,21,22,24</sup> The promoter regions for the OmpF and OmpC proteins consist of multiple protein binding regions, designated F1, F2, F3, F4, and C1, C2, C3 (Figure 1A), each approximately twelve nucleotides long. Two OmpR molecules bind to each site and then interact with the alpha subunit of RNA polymerase to control transcription.<sup>13</sup> Under conditions of low osmotic pressure, the nonphosphorylated form of the protein predominates. This form of the protein binds to high affinity sites on the *ompF* gene (F1, F2) and thus causes expression of the OmpF porin protein. As the osmotic pressure of the environment increases, the level of the phosphorylated form of the protein (OmpR-P) increases, and additional low affinity sites are occupied (C1, C2, C3, F3, and F4) as the protein becomes more active.<sup>15,22,24</sup> Binding to the low affinity F4 site results in transcriptional repression of the OmpF protein, while binding to the low affinity sites on the *ompC* gene results in transcriptional activation of the OmpC protein.<sup>15</sup>

The second response regulator that we chose to study is the VanR protein. In the bacteria *Enterococcus faecium*, the response regulator VanR and its cognate histidine kinase VanS control transcription of the *vanRS* and *vanHAX* genes by binding to multiple promoter binding regions, much like the OmpR protein.<sup>20</sup> When transcription of the *vanHAX* gene cluster is activated, there is a reduction in the affinity of the glycopeptide antibiotics for the cell wall of the bacteria—conferring antibiotic resistance.<sup>26,27</sup> The regulatory regions of the *vanRS* and *vanHAX* genes contain the promoter binding regions R1, H1, and H2 (Figure 1B).<sup>20</sup> However, the effect of VanR binding to these promoter regions is not as well understood

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**Table 1. DNA Probe Sequences for VanR and OmpR Response Regulators**

promoter region	sequence <sup>a</sup>
Omp F1	5' ACT TTT GGT TAC ATA TTT 3'
Omp F1F2	5' ACT TTT GGT TAC ATA TTT TTT CTT TTT GAA ACC AAA TC 3'
Omp C1	5' ACA TTT TGA AAC ATC TAT 3'
Van H1	5' ATT TTT TAG GAA AAT CTC 3'
Van H2	5' TTT TCT TAG GAA ATT AAC 3'
Van R1	5' TCA TCT TAA GAA ATT CTT 3'

<sup>a</sup> All immobilized oligonucleotides have a 5'-thiol modification and (T)<sub>15</sub> spacer.

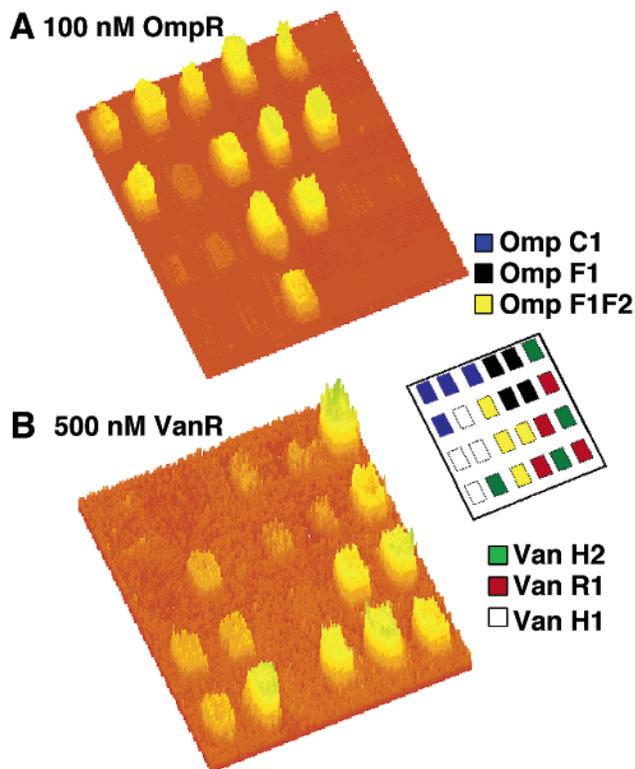
as the binding of the OmpR response regulator to the promoter regions of the *ompC* and *ompF* genes.

**B. DNA Array Fabrication for OmpR and VanR Binding Measurements.** A double-stranded DNA array consisting of six different sequences was constructed on a thin gold film to observe the binding of the OmpR and VanR proteins. Table 1 lists the sequences used in these studies. Three of the sequences (F1, F1F2, and C1) bind OmpR, and three of the sequences (R1, H1, and H2) bind VanR. The interaction of OmpR and VanR to these sequences has been studied previously using other methods.<sup>20,25</sup> For OmpR, the F1 and C1 sites were chosen over the remaining promoter elements (F2, F3, F4, C2, and C3) because it has been reported that the F1 and C1 sites must be present for protein to bind to the other sites.<sup>25</sup> The longer F1F2 region (53 bases) was chosen to determine whether a larger promoter region would affect protein binding.

Six single-stranded oligonucleotides were immobilized onto a gold thin film in the pattern shown in Figure 2. A polyethylene glycol-terminated surface separates each element to prevent the nonspecific adsorption of protein to the array background.<sup>10</sup> In addition to the protein binding region, each oligonucleotide was modified on the 5' end with a 15 base thymine spacer terminated with a thiol group for attachment to the surface. This spacer layer was required to prevent the DNA and protein from interacting with the gold surface and to provide room for the protein to interact with the binding region.

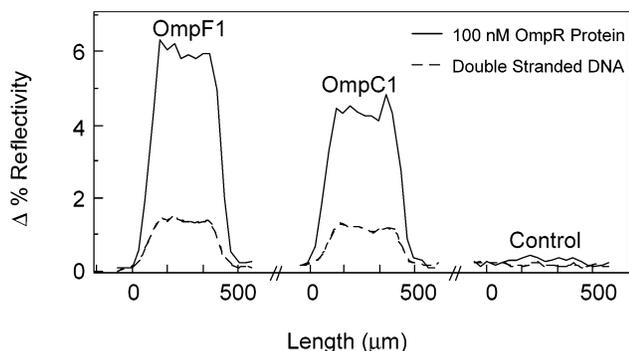
Since the OmpR and VanR proteins bind to double-stranded DNA (dsDNA), the single-stranded oligonucleotide array was converted to a dsDNA array through hybridization with complementary sequences. The amount of hybridization adsorption to the array varied with each complementary sequence. This variation is attributed to differences in oligonucleotide surface concentration and sequence-dependent hybridization efficiency. For example, Figure 3 shows the changes in the percent reflectivity (dotted lines) obtained by SPR imaging after exposing the F1 and C1 array elements to their complementary sequences. A control sequence (VanH1) was also included in the measurement. Hybridization was observed to both the F1 and C1 sequences, with 15% more hybridization occurring on the F1 array elements. Since these differences will affect the amount of protein that binds to the surface, the SPR signal that was obtained from this hybridization step was used to normalize signals in all of the subsequent protein binding measurements. No hybridization adsorption was observed to the VanH1 sequence after exposure to F1, F1F2, and C1 complements.

In comparison to the F1 and C1 sites, significantly lower amounts of hybridization were obtained with the F1F2 site than expected. This low surface concentration of double-stranded F1F2 DNA was attributed to accessibility problems due either to interactions with the surface or to the formation of secondary structure. The lower surface concentration of dsDNA resulted in a lower SPR signal from protein binding in subsequent steps.



**Figure 2.** Surface plasmon resonance difference images of a double-stranded DNA array after exposing it to (a) a 100 nM solution of the protein OmpR and (b) a 500 nM solution of the protein VanR. The surface was modified with the DNA sequences shown in the figure legend. The OmpF1, F1F2, and C1 sequences are known to bind to the OmpR protein, and the VanH1, H2, and R1 sequences are known to bind the VanR protein. Significant SPR signal is observed only for the response regulator binding to the sequences identified as known binding regions and not to the background or other sequences.

**C. SPR Imaging Measurements of OmpR and VanR Binding.** After construction of the dsDNA arrays, SPR imaging measurements were used to monitor the binding of OmpR and VanR. The SPR difference images shown in Figure 2 were obtained by subtracting the SPR images before and after exposing the array for 15 min to (Figure 2A) a 100 nM solution of the OmpR protein and (Figure 2B) a 500 nM solution of the VanR protein, both of which had been expressed and purified as GST fusion proteins. It is clear from the images that protein binding was observed at the promoter sequences corresponding to known binding regions and that there was very little nonspecific adsorption of the protein to the other sequences. A small amount of nonspecific adsorption was observed for the VanR protein toward the OmpF1 site; however, this binding was substantially smaller than the binding of VanR to any of its own promoter sequences. These measurements conclusively demonstrate that SPR imaging can be used to detect response regulator binding to target promoter regions immobilized on a DNA array fabricated on gold surfaces.

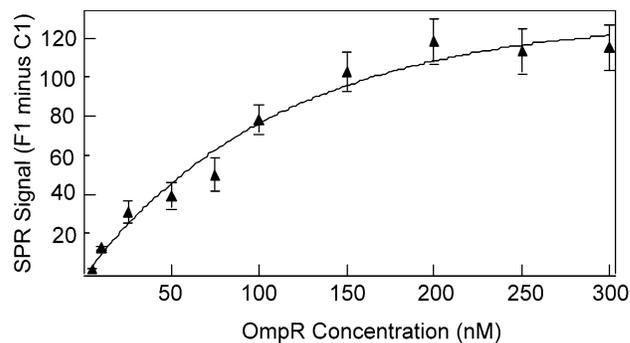


**Figure 3.** Plot profile showing the change in the percent reflectivity for the addition of DNA complement (dashed line) and the addition of 100 nM OmpR protein (solid line) for the indicated sequences, taken from the array shown in Figure 2A. The control was obtained on the VanH1 sequence with the addition of OmpF1, F1F2, and C1 DNA (dashed line), followed by hybridization to its complementary DNA strand (not shown), and finally 100 nM OmpR (solid line).

In addition to observing that the response regulators bound to their correct promoter sequences, we were interested in determining the amount of protein binding to a particular sequence relative to the other sequences. It can be seen from the images shown in Figure 2 that the SPR signal shows different amounts of protein binding to the different promoter fragments. It is possible to quantify the amount of protein binding to a DNA sequence by monitoring the changes in the percent reflectivity caused by protein adsorption to the array. This can then be compared to the change in percent reflectivity for protein adsorption to other sequences. Figure 3 shows line profiles constructed from the DNA array shown in Figure 2A. The signals from the F1, C1, and a control sequence for protein binding to the array (solid lines) have been plotted as a function of the change in the percent reflectivity and normalized to account for differences in surface concentrations of the immobilized oligonucleotides. These data show that at 100 nM OmpR concentration there is 25% more protein binding to the F1 sequence than the C1 sequence even after accounting for the fact that more F1 DNA hybridized to the surface. No significant signal was obtained from the control sequence. We have reported previously that the SPR imaging signal is linearly proportional to the amount of adsorbed material on a gold surface for changes in reflectivity up to 10%.<sup>9</sup> If quantitative data are desired for signals greater than this, corrections for nonlinear behavior must be made; however, qualitative data can be obtained in the nonlinear region without corrections.

The SPR imaging measurements can be used to study the amount of differential protein binding to the DNA arrays over a range of protein concentrations. For example, OmpR protein was observed to bind more strongly to the F1 site as compared to the C1 site for all concentrations from 1 to 300 nM. Figure 4 plots the difference in SPR signal due to OmpR binding for the F1 site over the C1 site as a function of protein concentration. These data agree with the reported observation that the F1 site is a higher affinity site than the C1 site in its nonphosphorylated state.<sup>15</sup> The concentration dependence of OmpR binding to the F1F2 sequence was not studied due to the secondary structure problems with the F1F2 DNA mentioned previously.

The range of protein concentrations over which OmpR and VanR binding to DNA arrays can be studied depends on a variety of factors. For very low protein concentrations, the number of protein molecules bound to a dsDNA array

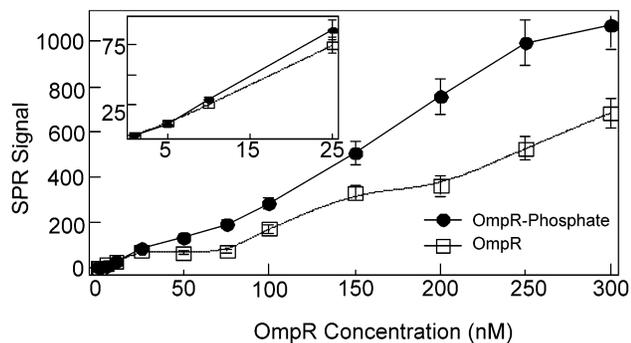


**Figure 4.** Graph showing the difference in the SPR signal for OmpR binding to the F1 and C1 sites obtained by comparing the plot profiles obtained for each site at the indicated concentrations. For all concentrations, more protein was observed to bind to the F1 site. The curve shown in the figure is an exponential fit of the data, which has been drawn to guide the eye.

element is determined by the protein–DNA binding constant. The lowest surface protein concentration that can be studied is determined by the detection limit of the SPR imaging technique. The combined effect of the binding constant value and the SPR imaging detection limit yields a bulk protein concentration detection limit, which for OmpR and VanR is approximately 5 and 50 nM, respectively. At higher protein concentrations, the fact that there is a finite number of available surface sites means that no changes in the SPR signal are observed above a certain bulk protein concentration. For OmpR and VanR, this limit was determined to be approximately 750 and 1250 nM, respectively. A second effect that occurs at high protein concentrations is the nonspecific adsorption of proteins to the array background and other array elements. Adsorption to the PEG background was observed for protein solutions above 750 nM; while in principle the SPR imaging measurements can be corrected for this effect, no SPR imaging data were used from these high concentration solutions.

**D. Measuring Changes in OmpR Binding with Protein Phosphorylation.** The control of gene expression by bacterial response regulators is directly related to the level of protein phosphorylation. Phosphorylation of OmpR induces a conformational change that makes binding to DNA 2–25 times more favorable than in its nonphosphorylated state.<sup>15,25</sup> This is controlled *in vivo*, by a cognate sensor kinase, such as the EnvZ histidine kinase associated with the OmpR protein. Many response regulators, including OmpR and VanR, can also be phosphorylated, both *in vivo* and *in vitro*, by a small molecule phosphodonor such as acetyl phosphate, carbamoyl phosphate, imidazole phosphate, or phosphoramidate.<sup>13,28</sup> Phosphorylation of OmpR by acetyl phosphate has been shown to have the same effects as phosphorylation by EnvZ.<sup>21</sup>

SPR imaging can be used to study the effects of protein phosphorylation on DNA binding. To show this, OmpR was phosphorylated (OmpR-P) with acetyl phosphate and exposed to a DNA array. Figure 5 shows the resulting SPR signal for OmpR-P (●) and OmpR (□) binding to the C1 promoter site for 1–300 nM protein concentrations. The isotherms for all OmpR binding sites were observed to level off near 75 nM and then increase at higher concentrations. For all concentrations above 5 nM, more SPR signal (up to 63%) was observed for OmpR-P adsorption to the C1 site than OmpR.

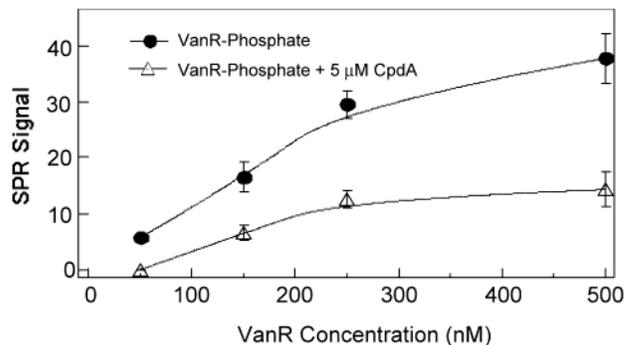


**Figure 5.** Graph showing the SPR signal obtained by integrating the area under the plot profile for the protein OmpR (□) or OmpR phosphorylated in solution with acetyl phosphate (●) binding to the OmpC1 DNA sites for the indicated protein concentrations. The figure inset shows the protein concentration range of 1–25 nM. Similar curves were obtained for the F1, F1F2 sites.

Phosphorylation of OmpR also increased the amount of adsorption to the F1 dsDNA array elements. We found that the C1 and F1 sites exhibited similar SPR imaging signals for low protein concentrations (1–150 nM), but at higher protein concentrations (200–300 nM), the OmpR-P binding to the C1 site was greater than the binding to the F1 site. This is in contrast to our differential binding results of the unphosphorylated OmpR protein, for which more protein was found to bind to the F1 site. Previous studies have reported that OmpR-P has approximately the same affinity for the F1 and C1 sites ( $K_D = 6$  nM).<sup>22,25</sup>

**E. Monitoring VanR Binding Inhibition by Small Molecules.** The binding of response regulators to promoter regions can be disrupted by small molecule binding inhibitors. There is interest in targeting two-component systems for broad-spectrum inhibition of virulent organisms.<sup>17</sup> Two-component systems have been directly linked to antibiotic resistance in certain bacteria,<sup>29</sup> and targeting these systems may yield useful therapies for treating antibiotic-resistant organisms. The response regulator/gene promoter binding interaction is one area within the two-component system amenable to attack by therapeutic agents.<sup>17</sup>

To demonstrate that SPR imaging can be used to screen small molecule DNA binding inhibitors, the binding of VanR protein was studied in the presence of a known DNA binding inhibitor of VanR. CpdA (2,3,4-trifluorophenylisothiazolone) has been shown to inhibit phosphoryl transfer from the VanS sensor kinase to VanR.<sup>30</sup> This compound also inhibits the binding of VanR-P and VanR to its DNA promoter sites. Figure 6 shows the resulting SPR signals for the binding of VanR-P in the presence (Δ) and absence (●) of 5 μM CpdA. Each data point is an average of the protein binding to three promoter sites on the DNA array (H1, H2, and R1). With these data, a very approximate  $K_d$  value of 150 nM, corresponding to the concentration where approximately 50% of the surface immobilized DNA binding sites are occupied, is obtained for the interaction of VanR-P binding to its promoter sites. Previous authors have used gel mobility shift assays to monitor the interaction of VanR-P binding to the entire *vanHAX* promoter region and have reported a  $EC_{50}$  value of 40 nM.<sup>20</sup> In the presence of 5 μM inhibitor, the SPR signal shows that there is 42% ± 4% less protein binding to the promoter sequences than without this compound



**Figure 6.** Graph showing the SPR signal obtained by integrating the area under the plot profile for the protein VanR-phosphate (●) or VanR-phosphate plus 5 μM DNA binding inhibitor CpdA (Δ) binding to promoter sequences for the indicated protein concentrations. The SPR signal is an average of the signal obtained for the three VanR DNA binding regions that were studied: VanH1, -H2, and -R1. More binding was observed for VanR-phosphate in the absence of inhibitor than VanR-phosphate with inhibitor present.

for all protein concentrations studied. The approximate value of  $K_d$  shifts to greater than 500 nM, assuming that the full coverage for VanR-P is unchanged. This shows that it is possible to monitor small molecule inhibition of response regulator protein binding to promoter sequences using SPR imaging. When the DNA array was exposed to VanR protein in the presence of 5 μM CpdA, no signal was observed for any sites at concentrations below 500 nM.

### Conclusions

In this paper, we have demonstrated that SPR imaging studies of DNA arrays fabricated on gold surfaces can be used as a label-free method for detecting the sequence-specific binding of transcription regulatory proteins. In the case of bacterial response regulators, SPR measurements can be used to identify and quantitate the high- and low-affinity binding sites within a gene's promoter region. These measurements will allow us to elucidate how response regulators control gene expression, and determine which binding sites act as repressors or activators of transcription. Monitoring variations in binding efficiency upon protein phosphorylation or in the presence of small molecule inhibitors can be easily obtained from SPR imaging measurements. The measurements on small molecule inhibitors suggest that this method will be generally useful in screening libraries of compounds for potentially therapeutic molecules.

The SPR studies reported here can be extended to other sequence-specific transcription regulatory proteins. For example, the sequencing of various bacterial genomes has identified over 250 different response regulator systems, and many of these have not been well characterized.<sup>31</sup> In addition, response regulators are just one specific type of transcription regulatory protein. There are many other classes of transcription factors in both prokaryotic and eukaryotic organisms, including, but not limited to, the Ets family of transcription factors<sup>32,33</sup> and the nuclear ligand-activated transcription factors, which includes steroid, thyroid, retinoid, vitamin D, and orphan receptors.<sup>34–37</sup>

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The technique of SPR imaging is not limited to small-scale DNA arrays, composed of only a few DNA sequences. It can also be combined with high-density DNA arrays for the identification of previously unknown transcription factors. Such complex arrays would be necessary to appropriately match transcription regulatory proteins

with the corresponding promoter regions of the regulated genes. SPR imaging measurements of high-density DNA arrays will be extremely useful in support of this effort to characterize DNA binding proteins whose sequences may be known but whose function remains unidentified.

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**Acknowledgment.** This research is funded by NSF (Grant CHE-9820742) and the NIH (Grant GM59622-02).

LA025609N