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Ligand Binding Kinetics of the Quorum Sensing Regulator PqsR

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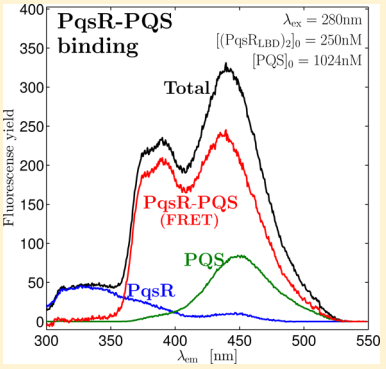
ABSTRACT: The *Pseudomonas aeruginosa* quinolone signal (PQS) is a quorum sensing molecule that plays an important role in regulating the virulence of this organism. We have purified the ligand binding domain of the receptor $PqsR_{LBD}$ for PQS and have used Förster resonance energy transfer fluorimetry and kinetic modeling to characterize the ligand binding *in vitro*. The dissociation constant for binding of PQS to a ligand binding site in $(PqsR_{LBD})_2$ dimers was determined to be 1.2 ± 0.3 μM. We found no cooperativity in the consecutive binding of two ligand molecules to the dimer.



Many species of bacteria use quorum sensing (QS) as a means of cell−cell communication.[1] QS is a mechanism that allows the cells within a culture to coordinate gene expression with the population cell density. Some species of Gram-negative bacteria employ *N*-acylated homoserine lactones (AHLs) as QS signals. These molecules are made and secreted by all members of the population and accumulate in the culture medium as a function of population cell density. Once a certain threshold AHL concentration is exceeded (i.e., the population becomes "quorate"), the AHL signals are thought to bind to cognate intracellular receptors. These receptors are transcriptional regulators that become activated or inhibited (depending on the protein) upon binding the ligand, allowing the resulting ligand−receptor complexes to modulate the expression of target genes. Thus, at high cell densities, the target genes are regulated synchronously across the whole bacterial population.[1] It is still not clear what determines the concentration of the signal molecule at which the population becomes quorate because a number of additional factors also impinge upon QS-dependent gene expression. For example, if the growth medium is changed, the amount of QS signal required to elicit a transcriptional response often changes. However, at the most basic and invariant level, the concentration of the QS signal required to achieve a "quorum" must be determined by the affinity of the receptor for the ligand.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen. It usually causes chronic and eventually fatal pulmonary infection in individuals with the genetic disease cystic fibrosis.[2] *P. aeruginosa* utilizes two quorum sensing networks dependent on AHL signals, similar to those of other Gram-negative bacteria.[3−5] *P. aeruginosa* also possesses a third quorum sensing system dependent on alkyl quinolones rather than AHLs as signaling molecules. The main quinolone signal molecule is 2-heptyl-3-hydroxy-4-quinolone, termed the *Pseudomonas* quinolone signal (PQS).[6] Like AHLs, PQS accumulates with increasing cell density and signals through its cognate receptor PqsR once the quorum is reached.[7,8] Loss-of-function mutations in PqsR or in the PQS biosynthetic genes diminish *P. aeruginosa* virulence.[9,10] Therefore, PQS-dependent quorum sensing represents an attractive target for therapeutic intervention in *P. aeruginosa* infections.[11]

PqsR is a LysR-type transcriptional regulator with an N-terminal DNA binding domain and a C-terminal ligand binding domain.[12] In the presence of PQS, PqsR binds strongly to target promoters,[13,14] likely as a homodimer. Previous workers have presented qualitative evidence that PqsR binds directly to PQS, but the assay used did not allow quantitative analysis of ligand binding.[12] In this report, we show that Förster resonance energy transfer (FRET) provides a convenient means by which to monitor binding of the ligand to the $PqsR_{LBD}$. In addition, we develop a detailed kinetic model for the PQS−$PqsR_{LBD}$ interaction, allowing us to extract quantitative binding data from the experimental results. Our approach may prove to be useful for quantifying the activity of compounds that interfere with PQS signaling.

METHODS

Expression and Purification of $PqsR_{LBD}$. The C-terminal region of PqsR (residues 91−242) encompassing the ligand binding domain (LBD) was expressed with a cleavable N-terminal maltose binding protein (MBP) affinity tag from

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plasmid pMalC2X in *Escherichia coli* strain CC118λpir. Cultures (2 × 500 mL volume) were grown with good aeration at 37 °C to an OD_{600} of 0.5 in Luria broth (LB). Following this, expression of the protein was induced by addition of isopropyl thiogalactopyranoside (IPTG, final concentration of 1 mM) for 2 h. The cells were pelleted (7500*g* at 4 °C for 10 min) and resuspended in buffer A [50 mM Tris-HCl, 0.3 M NaCl, 1 mM EDTA, 3 mM DTT, and 5% (v/v) glycerol (pH 7.5)] containing a cocktail of protease inhibitors (Roche). The cell suspension was lysed to completion on ice using a tip sonicator (maximal power output) and clarified by centrifugation (25000*g* at 4 °C for 20 min). The clear supernatant was loaded at a flow rate of 1 mL/min onto an amylose column (NEB) equilibrated with buffer A, and the column was washed overnight with ∼500 mL of the same buffer. The MBP-tagged protein was eluted with a small volume (∼10 mL) of buffer A containing 10 mM maltose. Factor Xa was added to the protein sample to cleave the MBP tag, and after being incubated for 2 h on a roller at 4 °C, the mixture was loaded onto a Q-Sepharose column equilibrated in buffer B [20 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, 3 mM DTT, and 5% (v/v) glycerol (pH 7.5)]. The column was washed overnight with buffer B, and the sample was eluted by increasing the salt concentration to 0.2 M. The eluted protein was loaded straight back onto an amylose column to capture the liberated MBP and any uncleaved MBP fusion protein. The flow-through was collected and concentrated. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of the samples revealed significant residual contamination of the 27 kDa $PqsR_{LBD}$ protein with the 42 kDa MBP protein. Therefore, the concentrated sample was loaded onto a Superdex 75 column equilibrated in buffer B. The $PqsR_{LBD}$ eluted at a position between that expected of a dimer of $PqsR_{LBD}$ and that expected of a trimer of $PqsR_{LBD}$, indicating that the $PqsR_{LBD}$ protein is probably a dimer. To fully resolve the $PqsR_{LBD}$ from residual contaminating MBP, the $PqsR_{LBD}$ peak fractions were collected, reconcentrated, and run again on the same column yielding a single isomeric peak (Figure 1). SDS−PAGE analysis of the peak fractions revealed that the $PqsR_{LBD}$ sample contained a single protein (inset of Figure 1).

PQS Titrations. The fluorimetric recordings were conducted in Tris-HCl buffer (pH 7.5) containing 0.05% *n*-dodecyl β-D-maltoside (Sigma-Aldrich catalog no. D4641-16), which was added to prevent precipitation of PQS. The concentration of $PqsR_{LBD}$, $[(PqsR_{LBD})_2]$, was fixed at 250 nM. For the titrations, we prepared serial 2-fold dilutions of PQS[15−17] in dimethyl sulfoxide (DMSO). Aliquots (0.5 μL) of these stock solutions were titrated into the protein samples (final volume of 1 mL in a quartz cuvette), and the fluorescence spectra were recorded using an LS55 Perkin-Elmer fluorimeter. The scan parameters were as follows: excitation wavelength λ_{ex} = 280 nm, emission wavelength $\lambda_{em} \in [300\text{ nm}, 550\text{ nm}]$, full slit widths $w_{ex} = w_{em} = 5$ nm, and scan speed of 50 nm/min. The temperature was maintained at 30 °C using a thermostated cuvette holder. Spectra without regulator and ligand molecules were recorded for baseline subtraction. Because the detailed shape of the RS and R spectra depended on the DMSO content, spectra with the same concentration of DMSO were used when resolving the linear combination in eq 2 (below). This procedure was not needed for the S spectrum because its shape was stable at >0.2% DMSO. The temporal stability of the spectra at each PQS concentration was confirmed by repeating each scan at least twice.

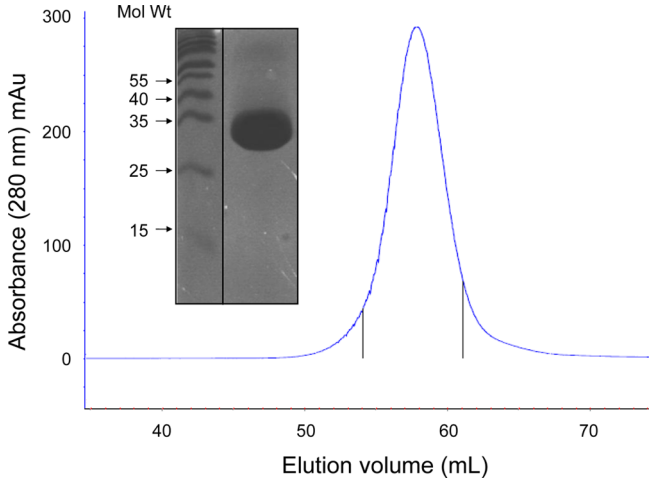

Figure 1. Elution profile of the ligand binding domain, $PqsR_{LBD}$, from the Superdex 75 column. Fractions between the vertical lines on the elution profile were pooled and concentrated for SDS−PAGE analysis. The inset shows SDS−PAGE analysis of the pooled and concentrated $PqsR_{LBD}$-containing fractions.

RESULTS AND DISCUSSION

FRET Titrations. The full-length PqsR protein was insoluble when overexpressed (data not shown). However, the ligand binding domain of the protein (residues 91−242 of the full-length protein, hereafter $PqsR_{LBD}$) was soluble and well-behaved in solution. The purified $PqsR_{LBD}$ migrated as a single band of ∼27 kDa on SDS−PAGE and eluted from a gel filtration column with a Stokes radius corresponding in size to that of a probable dimer of $PqsR_{LBD}$ (Figure 1). Preliminary crystallographic analyses are consistent with $PqsR_{LBD}$ being dimeric (T. Sams, B. F. Luisi, and M. Welch, unpublished data). The purified $PqsR_{LBD}$ contains two Trp residues and produced a strong fluorimetric signal at around λ_{em} = 340 nm when excited at λ_{ex} = 280 nm. Moreover, we found that the PQS ligand itself was fluorescent (even in the absence of protein). Conveniently, we found that when λ_{ex} was 340 nm, a robust fluorescence signal was observed at around λ_{em} = 450 nm. This observation immediately suggested that FRET might be used to monitor binding of the ligand to the protein. Indeed, we found that the PqsR−PQS complex formed a distinct coupled spectral state because of FRET and that the intensity of this spectral signature increased (up to a point) as the ligand concentration increased (Figure 2). No FRET was observed when $PqsR_{LBD}$ was substituted with lysozyme, indicating that the fluorescence signal was unlikely due to a nonspecific interaction of the PQS with the protein. We also found that in the absence of $PqsR_{LBD}$, the fluorescence yield scaled in direct proportion to the concentration of PQS added. This indicated that the fluorescence of PQS was not affected by the buffer components (or by trace contaminants in the buffer such as iron[18]). The distinct FRET observed between $PqsR_{LBD}$ and PQS suggested that we might be able to estimate the population of the different states (bound vs free ligand and protein) by measuring the fluorescence yield of PQS (S), $PqsR_{LBD}$ (R), and the two components mixed together at λ_{ex} = 280 nm. Notably, this could be done without any use of extrinsic fluorescence markers.

When the fluorimetric response of the $PqsR_{LBD}$ to increasing concentrations of PQS was measured, it appeared that the total yield could, to a good approximation, be written as a sum of

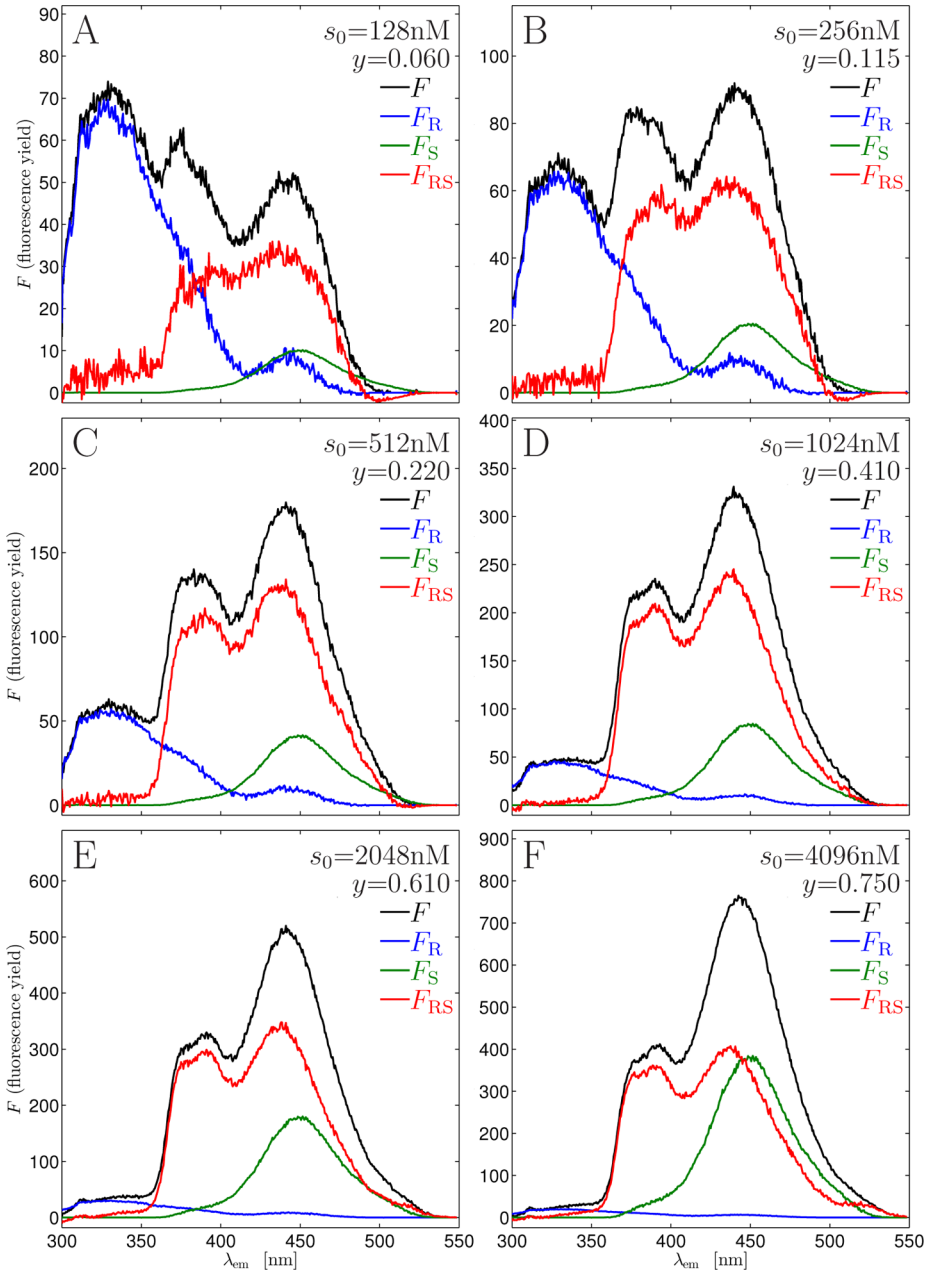

Figure 2. Deconvoluted fluorescence spectra at different concentrations of added PQS, where $s_0 = [S]_0$. The figure shows the RS (red) fluorescent yield deduced by subtracting the S (green) and R (blue) components weighted as in eq 2 from the total yield (black). The $PqsR_{LBD}$ concentration, r_0 (=2$[R_2]_0$), was 500 nM, and the excitation wavelength, λ_{ex}, was 280 nm. The PQS concentrations and corresponding occupancies in the panels were as follows: (A) s_0 = 128 nM and y = 0.060, (B) s_0 = 256 nM and y = 0.115, (C) s_0 = 512 nM and y = 0.220, (D) s_0 = 1024 nM and y = 0.410, (E) s_0 = 2048 nM and y = 0.610, and (F) s_0 = 4096 nM and y = 0.750, respectively.

three independent spectra, each representing one of the components. Because (based on the gel chromatography elution profile) the $PqsR_{LBD}$ was initially in the form of a dimer, this suggested that the fluorescence yields from the two presumed binding sites were independent. This is a fair assumption, provided that the two inferred ligand binding sites are sufficiently separated to avoid the formation of a coupled state. A model for consecutive ligand binding is shown in Figure 3. If we denote the fluorescence spectrum (normalized to concentration) as σ_S for free PQS molecules, σ_R for free PQS binding sites in either the free dimer (R_2) or partially occupied dimer (R_2S), and σ_{RS} for occupied PQS binding sites in R_2S or R_2S_2, then in terms of occupancy y of the PQS binding sites, the

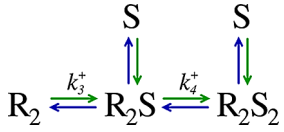

Figure 3. Binding model in which a preformed dimer of the ligand binding domain of PqsR consecutively binds two PQS molecules.

total fluorescence yield may be written as a linear combination of the three basic contributions:

$$F = F_{RS} + F_R + F_S \quad (1)$$

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$$F = y r_0 \sigma_{RS} + (1 - y) r_0 \sigma_R + \left(1 - \frac{r_0}{s_0} y\right) s_0 \sigma_S \quad (2)$$

where $r_0 = 2r_{20} = 2[R_2]_0$ and $s_0 = [S]_0$ are the initial concentrations of binding sites and ligands, respectively (Table 1 summarizes the kinetic constants and variables). As a result, the total spectrum is determined by a single parameter y at each ligand concentration.

Table 1. Symbols and Abbreviations

variable	details	explanation
F		total fluorescence yield
F_R		fluorescence yield from free PQS binding sites in $(PqsR_{LBD})_2$
F_S		fluorescence yield from free PQS
F_{RS}		fluorescence yield from occupied binding sites
k_3^+		on rate for binding of S to a single binding site in R_2
k_3^-		off rate for S in R_2S
K_3	k_3^-/k_3^+	dissociation constant for S in R_2S
k_4^+		on rate for binding of S to R_2S
k_4^-		off rate for a single S in R_2S_2
K_4	k_4^-/k_4^+	dissociation constant for a single S in R_2S_2
λ_{em}	300−550 nm	emission wavelength
λ_{ex}	280 nm	excitation wavelength
R	$PqsR_{LBD}$	ligand binding domain of PqsR
r_0	$2r_{20} = 2[R_2]_0$	total concentration of $PqsR_{LBD}$ counted as monomers
r_2	$[R_2]$	concentration of $PqsR_{LBD}$ dimers
r_3	$[R_2S]$	concentration of $PqsR_{LBD}$ dimers with a single PQS bound
r_4	$[R_2S_2]$	concentration of $PqsR_{LBD}$ dimers with two PQS molecules bound
S	PQS	ligand, signal molecule, 2-heptyl-3-hydroxy-4-quinolone
s_0	$[S]_0$	total concentration of PQS
s	$[S]$	concentration of free PQS
σ_R		concentration-normalized fluorescence yield for free binding sites
σ_{RS}		concentration-normalized fluorescence yield for occupied binding sites
σ_S		concentration-normalized fluorescence yield for free PQS
w_{em}	5 nm	full width of emission wavelength
w_{ex}	5 nm	full width of excitation wavelength
y	$(r_3 + 2r_4)/r_0$	occupancy of PQS binding sites

The calculated occupancy y at different PQS concentrations is given in Figure 2. At the lower end of the spectrum, only unoccupied ligand binding sites contribute to the spectra. Thus, $1 - y$ is the ratio between the total yield and the yield from the regulator alone. This leads to a robust determination at intermediate occupancy values, i.e., $0.25 < y < 0.75$. The remaining occupancies result from requiring the data collapse for the extracted F_{RS} when normalized to the occupancy ($F_{RS}/y = r_0\sigma_{RS}$), as shown in Figure 4. The consistency of the data collapse is a good confirmation that the total yield can indeed be written as the sum of the three independent spectra as indicated in eq 1. A plot of y versus s_0 yielded the titration curve shown in Figure 5. We shall now establish the formalism that allows us to interpret these data and extract the binding coefficient(s).

Development of a Kinetic Model for Binding of PQS to PqsR. The ligand binding domain of the PqsR regulator

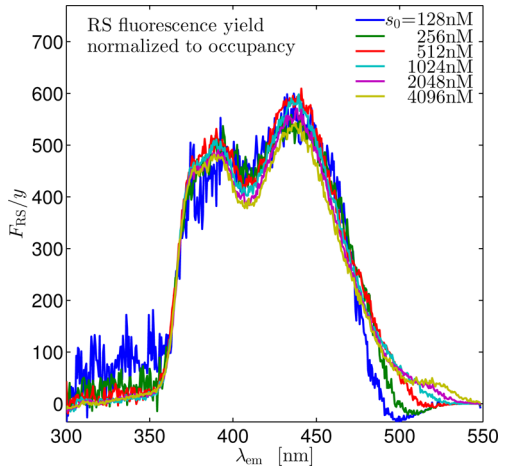

Figure 4. Consistency of data collapse. The figure shows the extracted RS fluorescence yield divided by the occupancy y of ligand binding domains.

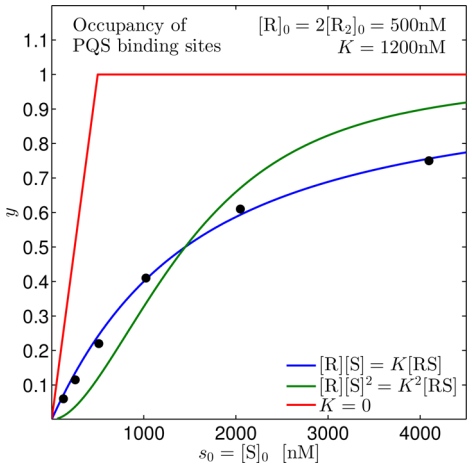

Figure 5. Titration of PQS into $(PqsR_{LBD})_2$ (i.e., $[R_2]_0 = 250$ nM). The ordinate is the deduced occupancy of PQS binding sites. Data are described well assuming no cooperativity ($h = 1$) and a dissociation constant K of 1.2 ± 0.3 μM (blue). For reference, also the curve corresponding to full cooperativity (green; $h = 2$) and the curve corresponding to the tightly bound ligand (red) are shown.

apparently forms a dimer even in the absence of the ligand. Assuming the simple case of consecutive binding of two ligands to the dimer (Figure 3), the kinetic equations are

$$\frac{dr_3}{dt} = 2k_3^+ r_2 s - k_3^- r_3 - k_4^+ r_3 s + 2k_4^- r_4 \quad (3)$$

$$\frac{dr_4}{dt} = k_4^+ r_3 s - 2k_4^- r_4 \quad (4)$$

where the combinatorial factor 2 is explicitly included. The coupling constants and variables are summarized in Table 1. With this normalization, $K_3 = K_4$ when there is no cooperativity in the consecutive binding of ligands to the regulator dimer. These equations are subject to the constraints

$$r_{20} = r_2 + r_3 + r_4 \quad (5)$$

$$s_0 = s + r_3 + 2r_4 \quad (6)$$

from conservation of regulator and ligand molecules. At static equilibrium

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$$2r_2 s = K_3 r_3 \tag{7}$$

$$r_3 s = 2K_4 r_4 \tag{8}$$

the distribution between dimer variants is

$$\frac{r_2}{r_{20}} = \frac{K_3K_4}{K_3K_4 + 2K_4 s + s^2} \tag{9}$$

$$\frac{r_3}{r_{20}} = \frac{2K_4 s}{K_3K_4 + 2K_4 s + s^2} \tag{10}$$

$$\frac{r_4}{r_{20}} = \frac{s^2}{K_3K_4 + 2K_4 s + s^2} \tag{11}$$

with

$$s = \left(1 - \frac{r_0}{s_0} y\right) s_0 \tag{12}$$

being the concentration of free ligands. The occupancy y of ligand binding sites may then be expressed as

$$y = \frac{[R_2S] + 2[R_2S_2]}{2[R_2]_0} = \frac{r_3 + 2r_4}{r_0} \tag{13}$$

$$y = \frac{s(K_4 + s)}{s(K_4 + s) + K_4(K_3 + s)} \tag{14}$$

After rearranging and simplifying we obtain

$$\frac{y}{1 - y} = \frac{s}{K_4} \frac{K_4 + s}{K_3 + s} \tag{15}$$

The log–log plot of $y/(1 - y)$ versus s shown in Figure 6 is a Hill plot.[19] It intersects with 1 at $s = K = (K_3K_4)^{1/2}$, and the slope h at this point is the Hill coefficient. In the case of no cooperativity ($h = 1$) and full cooperativity ($h = 2$) we obtain

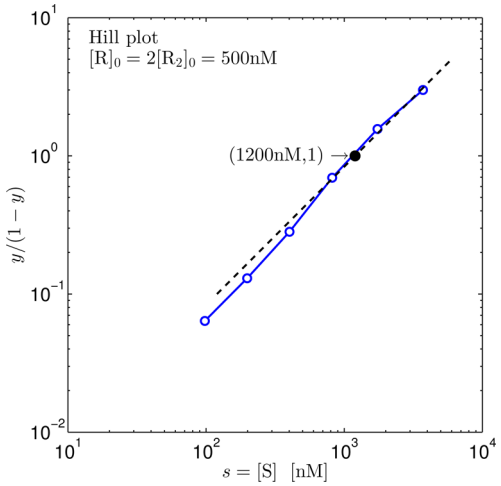

Figure 6. Hill plot. Half-occupancy is reached when $y/(1 - y) = 1$, and the corresponding concentration is the effective dissociation constant K. The slope of the curve at this point is the Hill coefficient. The dashed line indicates a Hill coefficient of 1, i.e., no cooperativity.

$$y = \begin{cases} \dfrac{s}{K + s} & h = 1,\ K_4 = K_3 = K \\ \dfrac{s^2}{K^2 + s^2} & h = 2,\ K_4 \ll K_3,\ K_4 < s < K_3 \end{cases} \tag{16}$$

For the sake of completeness, we also provide an expression (eq 17) for the concentration of the saturated dimer states R_2S_2 in the cases of no cooperativity ($K_4 = K_3$) and full cooperativity ($K_4 \ll K_3$). In our experiment, we were not able to observe this quantity directly.

$$\frac{r_4}{r_{20}} = \begin{cases} \dfrac{s^2}{(K + s)^2} & K_4 = K_3 = K \\ \dfrac{s^2}{K^2 + s^2} & K_4 \ll K_3,\ K_4 < s < K_3 \end{cases} \tag{17}$$

From Figure 6, we determined the dissociation constant to be $K = (K_3K_4)^{1/2} = 1.2 \pm 0.3$ μM and found no cooperativity in the consecutive binding of two ligands; i.e., $K_3 \approx K_4$. To the best of our knowledge, this is the first determination of the ligand binding properties of this important quorum sensing regulator.

Discussion. Previous workers have determined the concentration of PQS in cultures of wild-type *P. aeruginosa* (strain PAO1). In the earliest quantitation, Pesci et al. reported PQS concentrations of ~6 μM.[6] More recently, using a stable isotope dilution method, Lepine et al. found that PQS reaches a maximal concentration in the stationary phase of growth of ~16 μM.[7] Similar results were obtained by Diggle et al., who used TLC-based analyses to show that PQS concentrations reach 5–10 μM in the early stationary phase and reach ~25 μM in the late stationary phase.[20] Therefore, our determined K_d value is much smaller than the lowest of these estimates. However, it should be noted that PQS is a very hydrophobic molecule and these concentration estimates are for the total organic solvent-extractable PQS concentration present, much of which is likely packaged into membrane vesicles.[21] Indeed, the latter authors also showed that ~86% of the PQS present in a culture is vesicle-associated. The remainder is mostly cell-associated, so the K_d of the PqsR ligand binding domain for PQS (~1.2 μM) falls nicely between the upper and lower bounds of the probable cell-associated PQS concentrations, and in the most ligand-sensitive part of the titration curve. However, we also note that PqsR may be membrane-associated *in vivo*.[9] Presumably, this may further amplify the gain of the system if PQS is preferentially partitioned into this subcellular compartment because of its hydrophobicity (which, given its association with membrane vesicles, seems to be a reasonable assumption). Such partitioning could potentially increase the effective local concentration of PQS, a kind of zoning effect, allowing exquisitely sensitive titration of the ligand at low cell densities. Indeed, Xiao et al.[12] showed that the response of a *pqsA–lacZ* fusion to PQS was half-maximal around 0.04–0.4 μM.

In quorum sensing, the signaling organism is often described as responding to a critical "threshold" concentration of the signaling molecule, implying an "on–off" switch suggestive of cooperativity. However, if cooperativity is involved, it does not seem to be manifested at the level of ligand binding to its cognate receptor. We found no evidence of cooperativity in the binding of PQS to $PqsR_{LBD}$ and, furthermore, note that no cooperativity was observed in a previous study examining the binding of a different class of QS molecule [the QS signal in

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Pectobacterium carotovorum, *N*-(3-oxohexanoyl)-L-homoserine lactone] to its cognate LuxR-type receptor, CarR.[22] Indeed, the elegant transcriptome studies of Schuster et al.[23] indicate that perhaps we ought not to think of QS as being an on−off switch but, rather, as more of a rheostatic gene regulatory mechanism. If there is any "collectivity" in the PQS system, it probably stems from the positive feedback loop that controls PQS biosynthesis, rather than from the protein chemistry of ligand reception. Alternatively, the truncated form of the protein might not faithfully mimic ligand binding by the full-length species, a caveat that, given the extreme insolubility of the latter when it is overexpressed, may be difficult to prove.

Preliminary tests suggest that the FRET assay developed herein can also be applied to the study of the binding of HHQ to $PqsR_{LBD}$, which has been reported to be possibly complementary to PQS in *P. aeruginosa*.[8,12,24] HHQ is the precursor molecule of PQS and may play a significant role in signaling because of its solubility in aqueous solution being much higher than that of PQS,[8,24] although biologically, the molecule has been shown to be ∼100-fold less active than PQS.[12] The method for monitoring the binding of PQS to $PqsR_{LBD}$ introduced here can also potentially be applied to the study of the effect(s) of potential inhibitory compounds that might block or interfere with PQS reception.

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Notes

The authors declare no competing financial interest.

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