

Characterization of bacterial chemotaxis receptors sensing and signaling
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Abstract

Motile bacteria navigate in gradients of diverse chemical signals (toward attractants or away from repellents) by chemotaxis. Bacterial chemotaxis depends on a functioning signal transduction pathway comprising several cytoplasmic proteins that form a phosphorylation cascade. Chemotaxis signal transduction is initiated via sensing cues by dedicated chemoreceptors that are localized in the membrane [1]. The sensing activity of a bacterial chemotaxis receptor, named Tlp1 from *Azospirillum brasilense*, was recently shown to be regulated by binding to the second messenger molecule, c-di-GMP and a novel mechanism of sensory adaptation was proposed [2-4]. To further characterize this mechanism, a polyclonal antibody against the C-terminal region of Tlp1 was produced and purified. This antibody was then used to test whether it was able to detect the c-di-GMP bound or c-di-GMP free receptor in Western blot experiments, using the wild type strain and a mutant derivative over-expressing c-di-GMP. Although chemotaxis receptors are essential for bacteria to monitor their environments, the sensory specificity of most bacterial chemoreceptors encoded in completely sequenced genomes is largely unknown (88% of receptors have unknown function) despite the public databases harboring over 22,000 such chemoreceptors. Tlp2 is a chemoreceptor from *A. brasilense* with a periplasmic domain of unknown function. Genetic and behavioral evidence indicate that Tlp2 may specifically sense nitrogenous compounds (ammonium and nitrate) [5]. Isothermal Titration Calorimetry using a recombinantly produced sensing domain of Tlp2 as well as 3 of its variants produced by mutagenesis of conserved residues was used to further characterize the sensory specificity of Tlp2. These experiments demonstrated that Tlp2 is a novel sensor of nitrate.

Introduction

Chemotaxis

Chemotaxis is the movement of motile bacteria through a chemical gradient, towards attractants and away from repellents, respectively. Chemotaxis has been most thoroughly studied in the model bacteria, *Escherichia coli*. A reoccurring theme of chemotaxis is that a flagellar motor propagates bacterial movements through an aqueous environment characterized by random switches in rotational direction which lead the cells to ultimately reorient its direction of swimming. This rate of reorientation decreases as bacteria encounter attractants allowing them to move up a gradient of an attractant. The converse occurs in gradients of repellents. Commonly studied environmental factors that influence chemotaxis include pH, temperature, light, chemical concentrations, and gravity [6-12].

Chemoreceptor Architecture and Array

Chemotaxis receptor proteins are commonly referred to as chemoreceptors or methylaccepting chemotaxis proteins (MCPs). Chemoreceptors are comprised of 5 conserved domains (Fig 1): an N-terminal sensing domain, a transmembrane domain, a HAMP domain, and a highly conserved C-terminal signaling domain. The N-terminal domain functions as a sensory region; it is the most variable domain because it confers the chemoreceptor with the ability to sense various ligands in the bacteria's environment. A more conserved domain in chemoreceptor architecture is the HAMP domain, which, is named after the proteins that it is found in (histidine kinases, adenylate cyclases, MCPs, and phosphatases). The HAMP domain is a signal transduction module which converts the periplasmic signal received at the N-terminus of a protein to a cytoplasmic signal.

The most conserved region of the chemoreceptor is the methyl-accepting (MA) signaling domain, which interacts with downstream cytoplasmic chemotaxis proteins to initiate signal transduction [13].

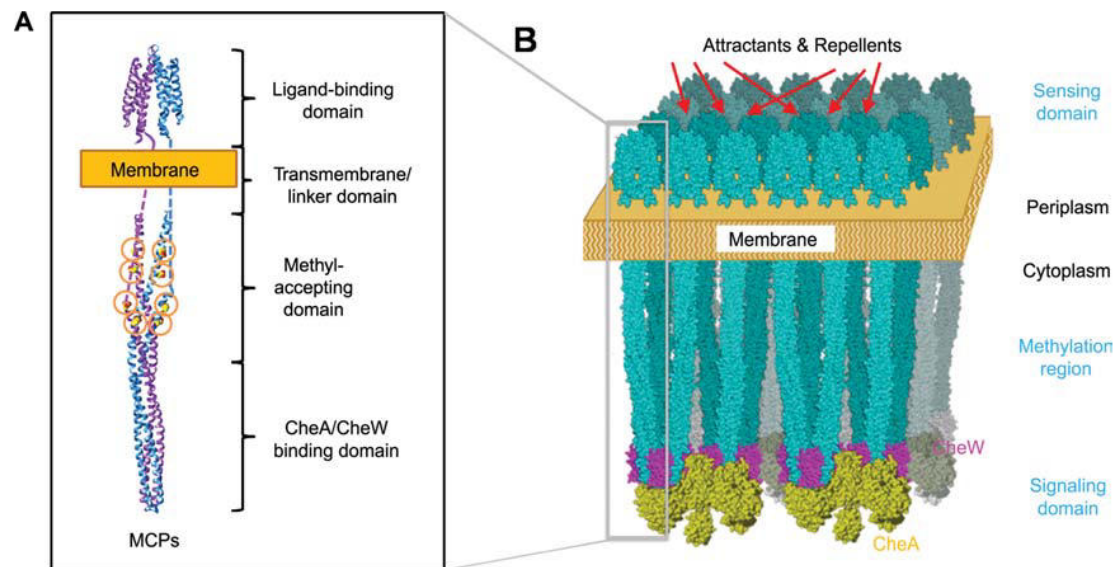


Figure 1: Architecture of chemotaxis receptors and chemoreceptors arrays. Reproduced from Li and Stock (2009) *Biol. Chem.* **390**: 1087-1096

c-di-GMP and the pilZ Domain

Cyclic dimeric GMP (c-di-GMP) is a ubiquitous second messenger in that has been known to regulate motility in diverse bacteria as well as cell surface association traits in bacteria. A dedicated c-di-GMP receptor is the PilZ domain, which is found as a single domain protein or combined with other protein domains in many species of bacteria. In the nitrogen fixing soil bacteria *Azospirillum brasilense* a PilZ domain is found at the extreme C-terminal region of the Tlp1 chemoreceptor [14].

Tlp1, a chemoreceptor with a PilZ domain

In *A. brasilense*, binding of c-di-GMP to the PilZ domain of Tlp1 modulates the ability of the chemoreceptor to adapt following sensing of a cue. In *A. brasilense*, Tlp1 functions to mediate the ability of the bacteria to respond and adapt to change in oxygen gradients (so called aerotaxis). Coincidentally, intracellular c-di-GMP levels are also regulated by changes in oxygen concentrations in *A. brasilense* where they signal transition from motility to sessility. Russell et al [15] have showed that sensing of c-di-GMP by Tlp1 allows *A. brasilense* to remain aerotactic under conditions where c-di-GMP levels are elevated and thus to persist as motile cells under conditions where they can generate maximum energy by following oxygen gradients.

Tlp2, a chemoreceptor of unknown function

Tlp2 is a chemoreceptor with a periplasmic domain of unknown function found in *A. brasilense*. Experimental evidence [5] suggests that this chemoreceptor functions to mediate chemotaxis in gradients of the nitrogenous compounds, nitrate and ammonium. This was demonstrated by intrinsic tyrosine fluorescence experiments, conducted by Dr. Matthew Russell. These findings have prompted further characterization of Tlp2's sensing specificity using more direct methods such as isothermal titration calorimetry.

Methods

Bacteria strains and growth conditions

The strains and plasmids used in this thesis are listed on Table 1. *E. coli* B834(DE3) were grown in 2xYT medium (Yeast Extract 16g^{-1} , Tryptone 10g^{-1} , NaCl 5g^{-1}) at 37°C at 225 rpm in 5 mL cultures. After growing overnight, these 5 mL cultures were used to inoculate two 2L flasks of

2XYT and grown at 37°C until reaching an OD⁶⁰⁰ of 0.6. Once the cultures reached OD⁶⁰⁰ of 0.6, they were induced with IPTG with a final concentration of 1mM. The following antibiotics were used at the listed final concentrations: Kanamycin (50 µg/ml) and Ampicillin (100 µg/ml).

Preparation of Cell Lysate

Cells were centrifuged at 8000 rpm for 20 minutes then resuspended with 20 mL of either RIPA (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris pH 8.0, PMSF) or lysis buffer (50 mM Tris pH 7.8, 250 mM NaCl, 10 mM Imidazole, 2 cOmplete ULTRA Tablets Mini (Roche)). Once resuspended, the cells were lysed with a sonic dismembrator 3 times for 30 seconds. The lysate was then homogenized by passing through a Thermo Electro Cop. French Press Cell Disrupter (Waltham, Mass.) at 25,000 psi. The lysate was then spun down at 17,000 rpm, for 1 hour, at 4°C.

Western Blot

After the samples had been run on a 12% Laemmli SDS-PAGE, the gel was soaked in transfer buffer (25mM Tris, 192 mM Glycine, 10% methanol). Proteins were transferred from the gel onto a nitrocellulose membrane using a Thermo Semi-Dry Transblotter for 20 minutes at 15 volts. The nitrocellulose membrane was incubated with 5% dry milk for 1 hour to block nonspecific binding and then rinsed with TTBS (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for 5 minutes, twice. After rinses with TTBS, the membrane was incubated with the primary affinity-purified Tlp1 antiserum at a dilution of 1:5000 overnight. The nitrocellulose membrane (Whatman 3M) was washed with TTBS three times for 5 minutes and incubated with ImmunoPure Rabbit IgG (H+L) antibody (Pierce) at a dilution of 1:10000 for 1 hour. Finally, the

membrane was washed with TTBS once for 20 minutes and then twice for 5 minutes. The membrane was then incubated with developing solutions (GE Amersham ECL Prime Western Blotting Detection Reagent) and ran through a film developer (GE).

Expression and purification of Tlp2 periplasmic domain

The DNA sequence coding for the ligand-binding domain of Tlp2 (Tlp2^{LBD}) was previously amplified by PCR and cloned into the pET-SUMO vector (Invitrogen) [5]. After being sequence-verified, a pET-SUMO containing the *tlp2*^{LBD} was transformed into *E. coli* B834 (DE3) (Novagen, Darmstadt, Germany). In the pET-SUMO vector, recombinant proteins are produced with a polyhistidine (6X His) tag at their N-terminus to facilitate protein purification by immobilized metal affinity chromatography (IMAC). The IMAC column (Kontes Flex-Column Economy Vineland, NJ) contained Ni-NTA agarose (Qiagen, Valencia, CA) and was washed with approximately 15 column volumes of wash buffer (50 mM Tris, 250 mM NaCl, 30 mM imidazol pH 7.8) prior to loading the cleared lysate. The lysate was loaded onto the column using either FPLC (GE AKTA Explorer 100) or gravity. Fractions were eluted from the column by running elution buffer (50 mM Tris, 250 mM NaCl, 300mM Imidazol, pH 7.8). These fractions were then analyzed by SDS-PAGE to ensure that the desired proteins were present and sufficiently pure (over 99%) for downstream analyses.

Characterizing the N-terminal sensing domain of Tlp2

Three mutants were created through site-directed mutagenesis using a commercial gene synthesis vendor (Genewiz), cloned in pET-SUMO vector, similar to *tlp2*^{LBD} (Campbell Reynolds, unpublished) and transformed into BL21(DE3) for protein recombinant expression. Conserved

arginine residues at positions 76, 139 and 159 were replaced by alanine to produce the following variants: Tlp2^{LBDR76A}, Tlp2^{LBDR139A} and Tlp2^{LBDR159A}. These proteins were expressed and purified by immobilized metal affinity chromatography in the same manner as described for Tlp2^{LBD}.

After being analyzed on a SDS-PAGE gel, the proteins were further purified using Size Exclusion Chromatography (GE HiPrep Sephacryl S-200 HR column with 20mM Tris, 150 mM NaCl, pH 7.8). The affinity for nitrate and ammonium was tested by isothermal titration calorimetry with a ligand concentration of 5mM NaNO₃ and a 5 mM ammonium chloride solution respectively in the same buffer as the protein (150 mM NaCl, 20 mM Tris, pH 7.8). The protein and ligand were loaded into the VP-ITC MicroCalorimetry machine and the data were interpreted using Origin-7.

Affinity purification of the Tlp1C antiserum

The crude Tlp1C antiserum (produced by immunizing rabbits with a recombinantly produced C-terminal signaling domain of Tlp1, named Tlp1C; Matthew Russell, unpublished) was first dialyzed against coupling buffer (0.1M carbonate buffer, 0.5M NaCl, pH 8.3). 10 mL of coupling buffer were added to 100µg of whole cell crude extract of the *Δtlp1* mutant strain of *A. brasilense*, produced by lysing whole cells using the BugBuster kit (Novagen) and incubated at 4°C over night. The next day, the beads were spun down for 2 minutes, at 1200 rpm and then washed 2 times with coupling buffer. The beads were resuspended in 15 mL of 0.1M TRIS-HCl, pH 8.0 to preserve the activity of the active groups and incubated for 2 hours at room temperature. The beads were washed 3 times at 4°C with 0.1M TRIS buffer (0.1M TRIS-HCl, 0.5M NaCl, pH 8.0) followed by 0.1M acetate buffer (0.1M Na acetate, 0.5M NaCl, pH 4.0). They were finally washed 3 times with PBS buffer (10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl,

pH 7.4). The crude Tlp1C antiserum was bound to the beads by resuspending 1 mL of beads treated as above with 10mM PBS in a 50mL Falcon tube. 10 ml of antiserum were added and incubated over night, at 4°C, on a tumbler. The next day, the beads were washed 3 times with PBS and resuspended in 10 mL PBS. The beads were then loaded into a BioRAD Poy-Prep chromatography column and washed with 10 volumes of PBS until OD 280nm = 0. The antibodies were eluted with 200 mM glycine pH 2.8 and 1 mL aliquots were collected into tubes containing 27 µL of 3M TRIS-HCL, pH 8.8 and 100 µL of 3M KCl (European Molecular Biology Laboratory).

Results

Purification of Tlp1 Antibody

As seen in (Figure 2) , affinity purification of the crude Tlp1C against a mutant strain of *A. brasilense* lacking Tlp1 ($\Delta tlp1$ strain) was successful in enhancing the specificity of the antiserum . Indeed, a protein band at about 70 kDa visible in the wild type strain and corresponding to the expected molecular weight of the full length Tlp1 is missing in the $\Delta tlp1$ mutant strains. Whole protein extracts from an *A. brasilense chsA::tn5* mutant strain, which lacks a major phosphodiesterase and was previously shown to contain elevated c-di-GMP levels [15] were used to test whether the antibody was sufficiently specific to detect differences in the c-di-GMP bound and unbound form of Tlp1. If this would be the case, we would expect to see a larger band or perhaps even more discrete bands in the region corresponding to Tlp1. As seen in (Figure 2), this was not the case.

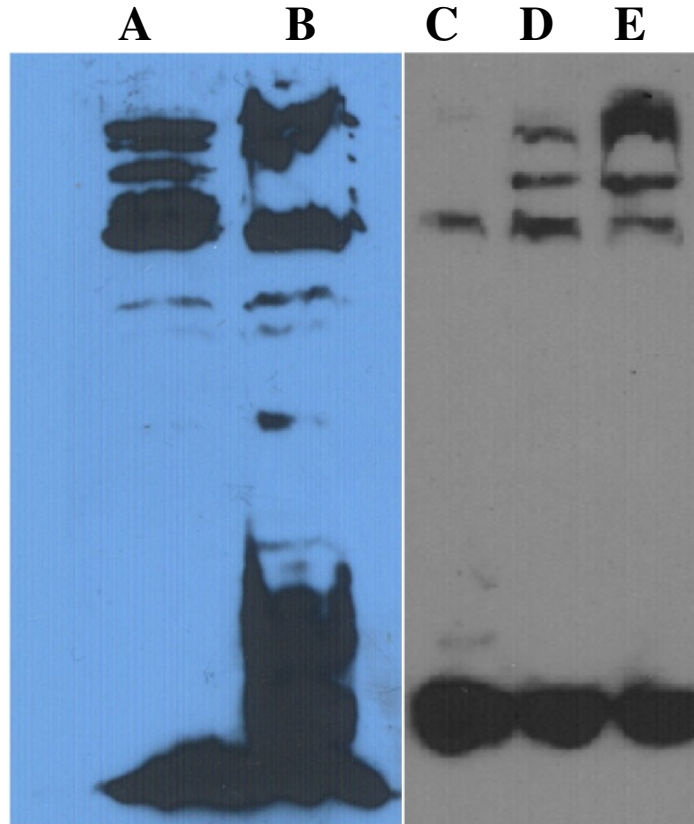


Figure 2. Western Blot of the Tlp1C antibody before purification (lanes A and B) and after purification (lanes C, D, and E). Lanes A and D contain whole protein extracts of the *A. brasilense* wild type Sp7 (lanes B and C), the Δ tlp1 mutant strain, (lane E), the *chsA::Tn5* mutant strain.

Characterization of Tlp2^{LBD} nitrogen-sensing domain

Isothermal titration calorimetry experiments allowed us to clarify the sensory specificity of Tlp2^{LBD}. Binding of Tlp2^{LBD} to nitrate (Figure 3) occurred with a relatively high affinity. Using Origin-70 software, it was determined that there was one nitrate binding site of physiological relevance within Tlp2^{LBD}, with a K_d of 2 μ M. When tested with ammonium, no binding was detected (Figure 4). When Tlp2^{LBD^{R76A}} was tested under similar conditions, it still was able to bind nitrate but with a significantly lowered affinity (K_d of 14 μ M).

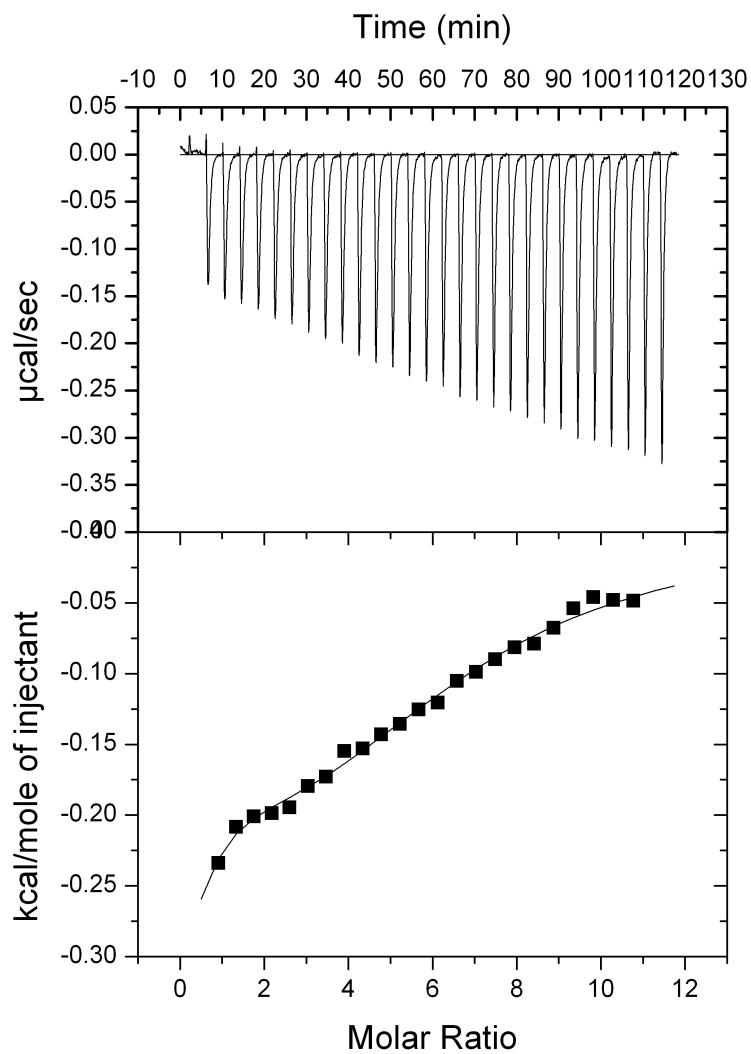


Figure 3. Binding of Tlp2^{LBD} to nitrate as determined by ITC.

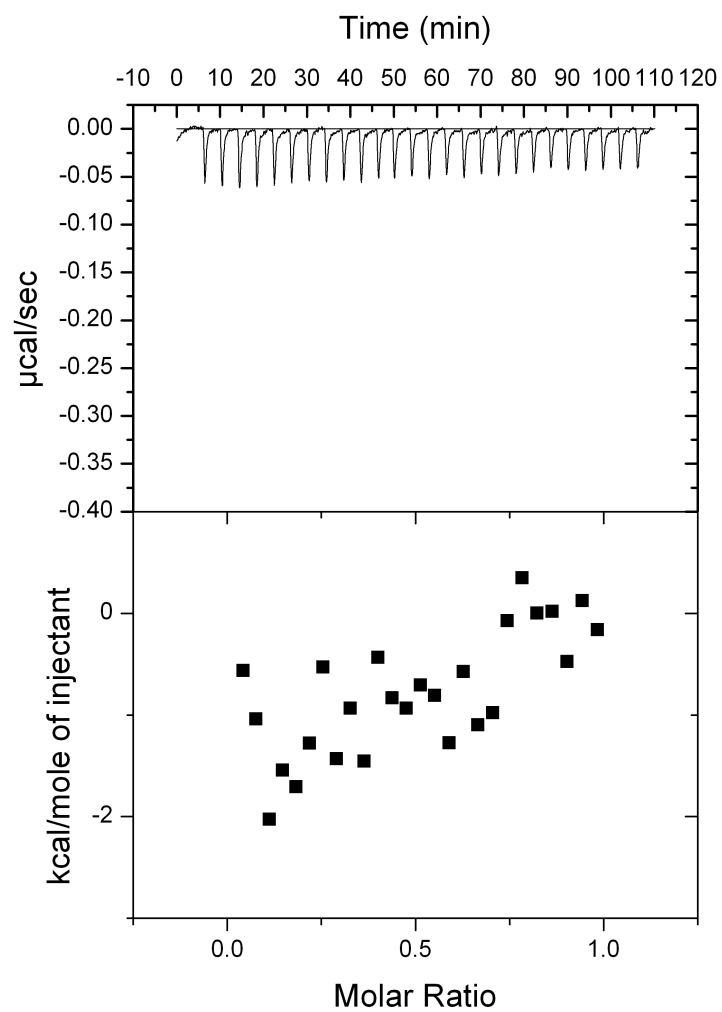


Figure 4. Binding of Tlp2^{LBD} to ammonium as determined by ITC.

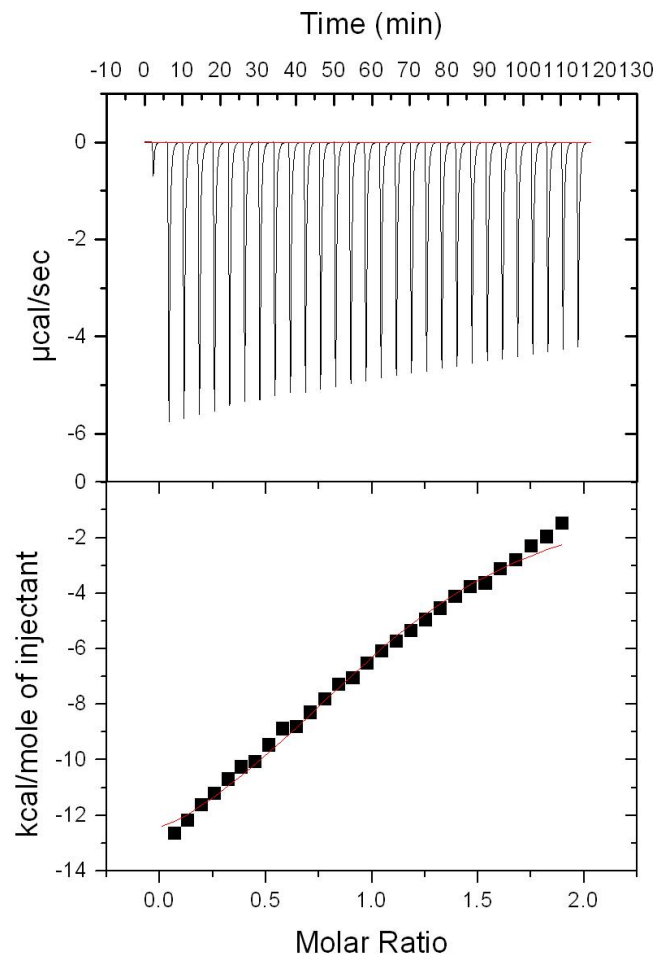


Figure 5. Binding of Tlp2^{LBD^{R76A}} to nitrate

Discussion

Affinity purification of Tlp1C antiserum and c-diGMP detection.

We were able to develop an affinity purified Tlp1C antiserum that had increased specificity for Tlp1. The antiserum, however, was still cross-reacting with additional proteins present in all strains tested. The very top bands seen in the wild type strain Sp7 and *chsA::Tn5* whole protein extracts may correspond to full length Tlp1 within the membrane since this band disappears in

the $\Delta tlp1$ mutant strain (Figure 2, panels CDE). The affinity-purified antiserum, however, was not able to discriminate between Tlp1 bound or unbound to c-di-GMP under the conditions used here. A more resolute PAGE gel or a continuous system could perhaps be used in the future to separate discrete bands of Tlp1, if they exist. Biophysical methods such as X-ray crystallography should also be pursued to characterize the molecular effects of c-di-GMP binding to the C-terminal signaling region of Tlp1 and to understand the molecular mechanism of c-di-GMP's affect on Tlp1's signaling activity.

Tlp2^{LBD} sensing specificity

Using sequential sites model, direct biophysical evidence has been obtained, supporting the role of the Tlp2's periplasmic sensing region as a novel nitrate-sensing domain. These findings are consistent with the genetic and behavioral evidence obtained thus far in the laboratory (Gladys Alexandre, unpublished). The isothermal titration calorimetry data did not support the existence of an ammonium binding site on Tlp2^{LBD}, which was suggested by, Dr. Russell's results using tyrosine fluorescence [5]. Such discrepancy is not unexpected given that tyrosine fluorescence is an indirect method to measure ligand binding to any protein. The ITC data obtained here are straightforward and clarified this finding. To further explore the novel nitrate sensing domain of Tlp2, variants carrying replacements of conserved arginine residues to alanine were constructed. ITC experiments performed thus far indicate that a mutation that replaces the conserved arginine 76 to an alanine residue causes a decreased affinity of the chemoreceptor to nitrate. Interestingly, the Tlp2^{LBD^{R76A}} is not null in nitrate binding, indicating that the conserved arginine 76 is likely contributing to protein structure but it is not an actual binding site for nitrate to Tlp2. Further studies with additional mutants where other conserved

arginine residues are replaced by alanine are currently underway. The findings of these experiments expand the repertoire of sensing abilities of chemotaxis receptors to include a nitrogenous ion. Our detailed molecular analysis paves the way to building a more complete understanding of how nitrate binds to the receptor to initiate signaling.

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