Lead(II) Detection

An Exceptionally Selective Lead(II)-Regulatory Protein from *Ralstonia Metallidurans*: Development of a Fluorescent Lead(II) Probe**

*Peng Chen, Bill Greenberg, Safiyh Taghavi, Christine Romano, Daniel van der Lelie, and Chuan He**

Lead contamination is a serious threat to human health and the environment.[1,2] Lead poisoning is still one of the most common environmentally caused diseases in the world today.[3,4] Lead levels are typically measured by using atomic absorption spectroscopy or other related instrumental methods. Probes that can provide rapid, on-site evaluation of the lead content of a sample are very valuable for a variety of applications.[5] A primary challenge is to construct a "turn-on" probe that responds to lead(II) ions with a high selectivity over other heavy metal ions. This has not been achieved with chemically designed small molecules, despite some progress in the last decade.[5] Chemical probes have been developed for the detection of other small molecules, but with limited success for heavy metal ions and paramagnetic metal ions.[6]

An evolution strategy was elegantly applied by Lu and coworkers recently to construct Pb^{2+} probes based on a DNAzyme.[7] This system is surprisingly selective towards Pb^{2+} ions. We have adopted a different strategy that takes advantage of the function of metalloregulatory proteins. Nature has evolved numerous such proteins to control the concentrations of beneficial or toxic metal ions with unprecedented sensitivity and selectivity. If the biological sensory events of these proteins were reported with measurable signals, such as fluorescence, practical probes could be obtained for the detection of various metal ions.[8–10] We have recently invented a method to convert the MerR-family proteins into fluorescent reporters for the detection of Hg^{2+} and coinage metal ions.[11] Herein we report the successful conversion of a novel Pb^{2+}-regulatory protein in *Ralstonia metallidurans* CH34 into a fluorescent reporter that exhibits high selectivity and sensitivity for Pb^{2+} ions. With the aid of this system, the binding of Pb^{2+} ions to the Pb^{2+}-regulatory protein was also characterized. A surprisingly high binding selectivity of the protein towards Pb^{2+} is revealed.

Ralstonia metallidurans is a Gram-negative, non-spore-forming bacillus that flourishes in millimolar concentrations of toxic heavy metals.[12] It is the only bacterium that has been shown to contain a lead-specific resistance pathway so far. The *pbr* operon is a unique operon, as it combines functions involved in uptake, efflux, and accumulation of Pb^{2+} ions.[13] All the resistance genes (*pbrTRABCD*) in the *pbr* operon are regulated by the PbrR protein, which mediates Pb^{2+}-inducible transcription from its divergent promoter (Figure 1). PbrR is

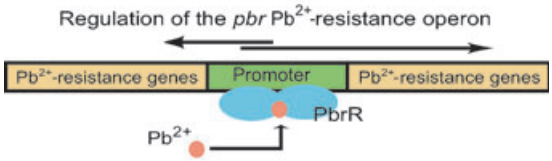

Figure 1. PbrR regulates the lead-resistance operon (*pbr*) in *Ralstonia metallidurans* strain CH34.

a member of the MerR family of metal-sensing regulatory proteins. It is the first protein to be discovered that can sense Pb^{2+} ions in nature.[13] PbrR691, another MerR-type protein that is encoded on the chromosome of *R. metallidurans* CH34, has been assigned as a homologue of PbrR (unpublished results);[14] however, its function has never been characterized. The binding of Pb^{2+} ions to PbrR and PbrR691 has not been studied in vitro. The concentration level of Pb^{2+} that triggers a response from these proteins is unclear. The selectivity and the molecular mechanism of the Pb^{2+} recognition have yet to be elucidated. We became very interested in studying these unique proteins, not only for developing Pb^{2+}-specific probes, but also to reveal strategies used by these proteins for recognizing trace levels of Pb^{2+} ions.

We applied a technique that we developed previously to convert the PbrR proteins into fluorescent reporters for Pb^{2+} ions.[11] The unique transcriptional activation mechanism used by the MerR proteins forms the basis for our approach.[15] A 25-mer duplex DNA containing the PbrR-binding sequence was prepared (**DNA-1** in Figure 2). In the central base pair of this sequence a fluorescent base, pyrrolo-C, was incorporated as the reporter. The addition of Pb^{2+} ions and PbrR should trigger a distortion of the duplex DNA to give an unpaired pyrrolo-C base, which will emit strong fluorescence at $\lambda \approx 445$ nm upon excitation at 350 nm. In the absence of PbrR and metal ions, **DNA-1** is expected to exhibit weak fluorescence.

The relevant genes for both PbrR and PbrR691 were cloned and expressed. PbrR showed a low solubility in aqueous solution. Fortunately, PbrR691 can be overexpressed as a soluble protein in large quantities. This protein was

[*] Prof. P. Chen, C. Romano, C. He
Department of Chemistry
The University of Chicago
5735 S. Ellis Avenue, Chicago, IL 60637 (USA)
Fax: (+1) 773-702-0805
E-mail: chuanhe@uchicago.edu
B. Greenberg, Dr. S. Taghavi, Dr. D. van der Lelie
Biology Department, Building 463
Brookhaven National Laboratory
50 Bell Avenue, Upton, NY 11973-5000 (USA)

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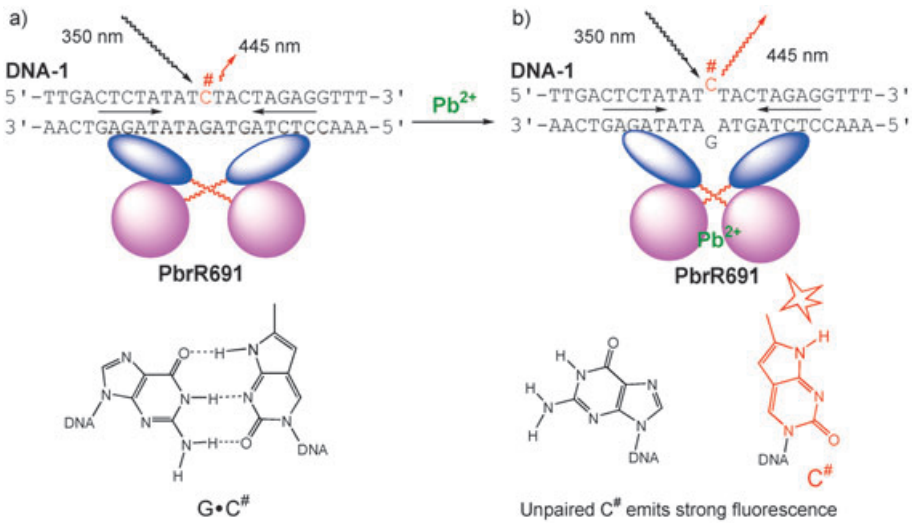

Figure 2. a) Pyrrolo-C ($C^{\#}$) can form a stable base pair with G. The fluorescence intensity of pyrrolo-C is quenched in the duplex DNA. The promoter sequence that PbrR691 binds is used to construct **DNA-1**; the dyad symmetrical sequence is marked with arrows. b) Binding of Pb^{2+} ions to PbrR691 induces base unpairing of pyrrolo-C, which emits strong fluorescence at $\lambda \approx 445$ nm upon excitation.

subsequently purified and used in this study (see the Supporting Information). The concentration of PbrR691 was determined by a bovine serum albumin (BSA) assay (Bio-Rad Laboratories, Inc.) that was calibrated by amino acid analysis (Protein Chemistry Laboratory, Texas A&M University).

The addition of PbrR691 to **DNA-1** did not cause noticeable changes in the fluorescence of the probe. However, the addition of one equivalent of Pb^{2+} ions per PbrR691 dimer triggered a significant fluorescence enhancement within seconds (Figure 3a). The probe is selective, as the addition of a 50-fold excess of Zn^{2+}, Co^{2+}, Hg^{2+}, Cu^{2+}, or Cd^{2+} caused less than 1/20th of the fluorescence increase at $\lambda = 445$ nm compared to that with Pb^{2+} ions (Figure 3b). We concluded that the fluorescence response of this PbrR691-based probe is over 1000-fold more selective towards Pb^{2+} ions than towards the other metal ions in this assay! Thus, PbrR691 appears to exhibit an unprecedented selectivity towards Pb^{2+} ions over other metal ions.

When we tried to perform an accurate analysis of Pb^{2+} binding to the protein by using this fluorescent system, we discovered that the fluorescence response from this probe is not optimum to give a quantitative assessment of Pb^{2+} binding to PbrR691. Instead, we employed 2-aminopurine (2AP), an analogue of adenine, as the fluorescent base. 2AP emits fluorescence at $\lambda \approx 370$ nm when excited between $\lambda = 310$ and 320 nm. The incorporation of 2AP into DNA quenches its fluorescence. This quenching is attributed to stacking interactions with nearest neighbor nucleobases and to electron transfer to nearby bases.[16,17] When the base-pairing environment is perturbed, 2AP shows quantitatively enhanced fluorescence; this property makes 2AP an excellent fluorescent probe for studying the structural properties of the DNA duplexes. We incorporated a 2AP base into the central base pair of the PbrR-promoter DNA (**DNA-2**), as shown in Figure 4a. The response of this new probe towards Pb^{2+} binding was tested.

The fluorescence of the 2AP-containing probe is quenched in double-stranded DNA. The addition of the apo-PbrR691 dimer to **DNA-2** did not give any significant fluorescence change. The addition of one equivalent of Pb^{2+} ions triggered a greater than threefold fluorescence enhancement. The intensity increase is reproducible and the response occurs at both 4 °C and room temperature. At room temperature, the detection limit can reach the nanomolar range (50 nM) for free Pb^{2+} ions in solution. The probe also shows higher selectivity towards Pb^{2+} ions than towards other metal ions (about 1000-fold, Figure 4b). The binding of Pb^{2+} ions to

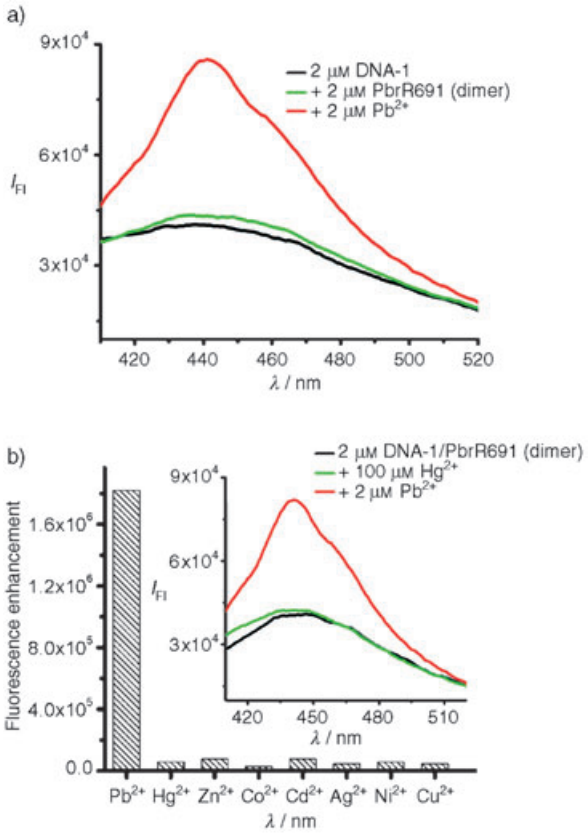

Figure 3. Responses of the PbrR691-based fluorescent probe towards different metal ions. a) Fluorescence spectra of the probe in the absence and presence of one equivalent of Pb^{2+} ions. b) The fluorescence enhancement integrated over $\lambda = 420$–500 nm in the presence of Pb^{2+} ions (2 μM) and other metal ions (100 μM). The inset presents the fluorescence spectra of the probe in the presence of Pb^{2+} (2 μM) and Hg^{2+} ions (100 μM).

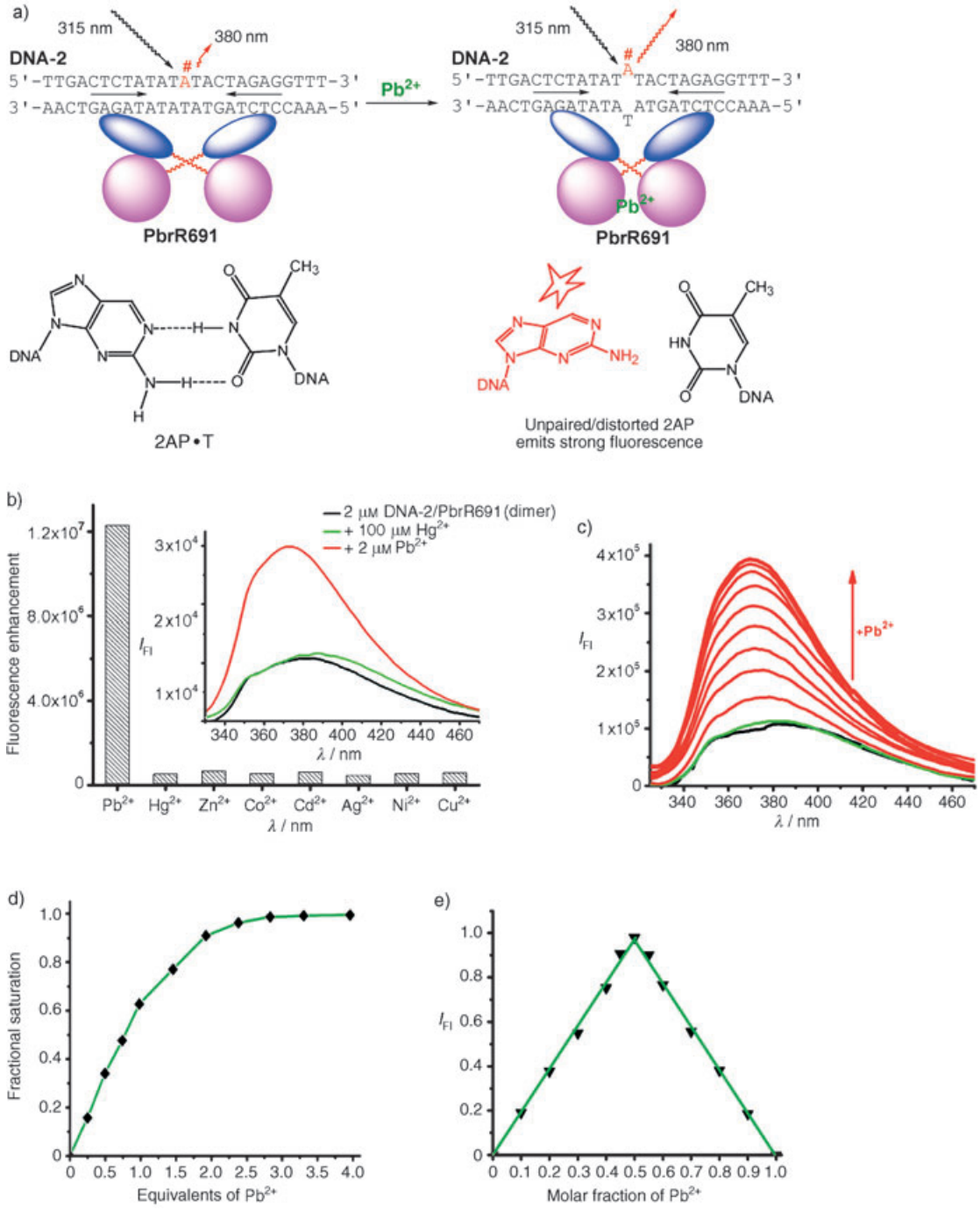

Figure 4. Binding of Pb^{2+} ions to PbrR691 as revealed by a 2AP-modified DNA probe (**DNA-2**). $A^{\#}$ = 2-aminopurine = 2AP. a) Binding of Pb^{2+} ions to PbrR691 induces base unpairing of 2AP, which emits strong fluorescence. b) The fluorescence enhancement integrated over $\lambda = 350$–420 nm in the presence of different metal ions. The insert presents the fluorescence spectra of the probe in the presence of Pb^{2+} (2 μM) and Hg^{2+} ions (100 μM). c) Fluorescence response of the PbrR691(dimer)/**DNA-2** complex to the addition of Pb^{2+} ions at pH 7.0. The initial concentrations of PbrR691(dimer) and **DNA-2** were both 2.0 μM (black: fluorescence of **DNA-2** only; green: fluorescence for the complex). Aliquots of 0.1 or 1 mM $Pb(NO_3)_2$ were added to yield final Pb^{2+} concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.5, and 10.0 μM (red curves). d) The fractional saturation of the Pb^{2+}/PbrR691(dimer)/**DNA-2** complex as a function of Pb^{2+} ions added, based on the spectra presented in (c). e) Job plot of Pb^{2+} binding to PbrR691. The DNA concentration (**DNA-2**) was maintained at 4 μM. The sum of the concentration of PbrR691 and $Pb(NO_3)_2$ is 4 μM.

PbrR691 appears to be reversible, as the addition of 10 μM ethylenediaminetetraacetate (EDTA) to the Pb^{2+}-bound probe restores the original spectrum of the metal-free probe.

This new 2AP-based Pb^{2+} probe has several advantages. It is sensitive and highly selective towards Pb^{2+} ions. It shows a quantitative response to various Pb^{2+} concentrations in solution. The fluorescent-reporter system also offers an opportunity to characterize the Pb^{2+}-binding properties of PbrR691. The binding of Pb^{2+} ions to PbrR691 was monitored by recording fluorescence spectra as a function of Pb^{2+}

concentration, as shown in Figure 4c. The metal-binding titration was fitted with a one-site binding model in the Origin program (Origin 7 SR2, OriginLab Corp., Northampton, MA). The fitting curve yielded a dissociation constant, K_d, of $1.98 \pm 0.16 \times 10^{-7}$ M (Figure 4d). The fitting also indicated that PbrR691 forms a 2:1 complex with Pb^{2+} ions; thus, each PbrR691 dimer binds one Pb^{2+} ion.

To confirm the binding constant and the stoichiometry of the metal–protein complex in solution, we performed the continuous variation experiment (Job method; see the Supporting Information),[18] which unambiguously confirmed the formation of a 2:1 PbrR691:Pb^{2+} complex (Figure 4e). This conclusion agrees with the fluorescence titration result. To verify the binding constant, isothermal titration calorimetry (ITC) was performed on the Pb^{2+}/PbrR691 system in a solution of 100 mM $NaNO_3$, 20 mM tris(hydroxymethyl)aminomethane/HNO_3 (Tris-HNO_3), and 5% glycerol at pH 7.0.[19] The ITC data were fitted by the one-site binding model and they yielded the same 2:1 ratio between PbrR691 and Pb^{2+} ions (see the Supporting Information). The dissociation constant was calculated to be $2.07 \pm 0.14 \times 10^{-7}$ M, which is consistent with the fluorescence titration result.

Lastly, the Pb^{2+}-bound PbrR691 was prepared and analyzed. Excess amounts of $Pb(NO_3)_2$ (up to a final concentration of 500 μM) were added to a solution of PbrR691 (50 μM). The free and weakly bound Pb^{2+} ions were removed by consecutive dialyses with washing buffer (2 L). Inductively coupled plasma-mass spectrometry (ICP-MS) analysis of this sample showed 1.06 ± 0.02 equivalents of Pb^{2+} ions per PbrR691 dimer. When the protein solution was washed with EDTA (100 μM), almost no Pb^{2+} ions (0.008 equiv) were observed from the ICP-MS measurement. This observation further indicates the binding of one Pb^{2+} ion per PbrR691 dimer.

We have reported herein the characterization of a unique Pb^{2+}-regulatory protein, PbrR691. It shows a selectivity of over 1000-fold towards Pb^{2+} ions over other metal ions. By applying a mechanism-based approach, we have successfully converted this Pb^{2+}-sensory protein into a selective fluorescent Pb^{2+} reporter. This novel PbrR691-based Pb^{2+} probe has the potential to be used in practical applications after further improvements. The affinity and stoichiometry of the binding of Pb^{2+} ions to PbrR691 were also revealed with the aid of the fluorescent-reporter system and were further confirmed with other methods.

The development of a selective Pb^{2+} chelator for treating lead poisoning is an unachieved challenge.[1] We believe study of the molecular basis for selective Pb^{2+} recognition by PbrR691 may provide fundamental knowledge for designing such agents in the future.

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