Inorganic Chemistry

Communication

pubs.acs.org/IC

Smart "Lanthano" Proteins for Phospholipid Sensing

Shafali Gupta,[†] Samsuzzoha Mondal,[†] Amit Mhamane,[‡] and Ankona Datta*

Department of Chemical Sciences, Tata Institute of Fundamental Research, 1 Homi Bhabha Road, Colaba, Mumbai 400005, India

Supporting Information

ABSTRACT: Metal-ion-mediated interactions between calcium-binding peripheral proteins and membrane phospholipids is a key feature of multiple cell signaling processes. The molecular basis for the interaction involves the displacement of inner-sphere water molecules on calcium ions by phosphate groups of the phospholipids. On the basis of this fundamental mechanism, we have devised a novel "turn-on" optical sensing strategy for anionic phospholipids by using a lanthanide reconstituted protein. The "lanthano" protein turns on selectively in the presence of a crucial signaling phospholipid, phosphatidylserine, by affording a 6 times enhancement in lanthanide luminescence. The "turn-on" sensing strategy was distinctly validated by direct evidence for the water-displacement mechanism via lifetime measurements.

Cell membrane phospholipids (PLs) are key effectors of biological signaling processes.[1] Quintessential cell signaling pathways are mediated via membrane interacting peripheral proteins that have specific interactions with PLs.[1,2] Hence, PL sensing via appropriately modified proteins can afford crucial diagnostic and mechanistic information on the physiological state of a cell.[3] A common strategy for PL detection involves the use of protein−dye conjugates incorporating fluorophores ranging from fluorescein to green fluorescent protein.[3a,b,4] However, a strong background signal from unbound sensors is a major shortcoming of current PL detection assays.[5] Consequently, PL assays are used for imaging in fixed tissues with restricted live, real-time imaging applications, notable among which is recent work on zinc-based sensors.[3b,6] The issue of high background can only be addressed by developing "turn-on" sensors that can give a specific read-out upon interaction with membrane PLs.

Peripheral proteins, such as protein kinase C and annexins, are often exploited for PL sensing.[1] Ca^{II}-dependent PL binding, in which the metal ions are simultaneously coordinated to the protein ligands and the polar PL headgroups, has been implicated in the membrane-binding mechanism.[2,7] Building upon this special PL−metal ion interaction, we have developed a unique "turn-on" detection strategy for anionic PLs via lanthanide (Ln) reconstituted proteins or "lanthano" proteins. The anionic PL selected for this study is phosphatidylserine (PS), an essential lipid involved in protein recruitment to the plasma membrane.[1b] More importantly, PS, normally present in the inner leaflet of the plasma membrane, is transferred to the outer layer during early stages of apoptosis.[3c,4,5,6b,c,8] As a proof-of-concept toward "turn-on" PL detection mediated by "lanthano" proteins, we have chosen to sense the interaction of this essential signaling PL with a peripheral protein annexin V (Anx V).

Our protein of choice, human Anx V, is a 36 kDa protein implicated in anticoagulation and has nanomolar affinity (K_d = 0.1−2 nM) toward PS in the presence of millimolar levels of Ca^{II}.[3c,7d,9] Crystal structures of the protein sans PL have identified five Ca^{II} binding sites (Figure 1a).[10] The current

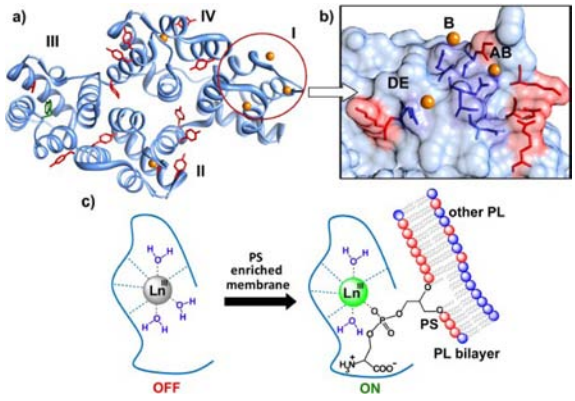

Figure 1. (a) Crystal structure of Human Anx V (PDB: 1AVR) depicting Ca^{II} sites (golden) with aromatic residues highlighted (Y: red; W: green). (b) Three Ca^{II} binding sites (AB, B, and DE) of domain I indicated with residues interacting with PS carboxylate (red) and residues interacting with PS amine (blue).[7e] (c) "Turn-on" sensing strategy for PS detection via "lanthano" AnxV.

consensus molecular mechanism for PS binding to Anx V implicates coordination of PS phosphate groups to the Ca^{II} ions (Figure 1a,b) of the protein, via the displacement of inner-sphere water molecules.[7c−e,11] Strategically placed amino acids with positive and negative side chains, shown in Figure 1b, interact with the carboxyl and amine moieties of the serine headgroup.[7e] The interactions presented by these residues provide the specificity for PS; highlighting the importance of molecular recognition along with electrostatics.[7d,12]

We envisioned that the displacement of coordinated water molecules on Ca^{II} ions by PS could be translated to an appropriate "turn-on" sensing strategy by substituting the metal ions with lanthanides (Figure 1c). Lanthanides can be sensitized to luminesce via energy transfer from aromatic chromophores, and the luminescence is quenched by coordinated water molecules.[13] Furthermore, Ln^{III} ions (Ln^{III}) have size and preference toward oxygen donor atoms similar to those of Ca^{II}. Anx V has ample aromatic amino acids (Figure 1a) for Ln

Received: July 23, 2013
Published: October 15, 2013

   dx.doi.org/10.1021/ic4018932 | *Inorg. Chem.* 2013, 52, 12314−12316

sensitization, and prior reports indicate the affinity of Ln^{III} to Anx V Ca^{II} sites.[10] We thus hypothesized that Ln-reconstituted Anx V would be ideal for developing "turn-on" PS sensors. The initially quenched Ln^{III} luminescence would increase upon PS binding because of the displacement of inner-sphere water molecules (Figure 1c). Modification in the environment of the aromatic amino acids upon PL-protein binding might also marginally alter Ln sensitization.[13c] More importantly, "lanthano" Anx V would show similar specificity for PS binding because interactions with the anionic phosphoserine headgroup would essentially remain unperturbed.

In order to test our "turn-on" sensing strategy, Anx V was expressed and purified, followed by desalting to remove metal ions (see the Supporting Information, SI). Inductively coupled plasma optical emission spectroscopy on the protein indicated the presence of minimal 0.1 calcium per protein molecule. The purified protein was reconstituted with Ln^{III} to prepare the PL sensor. Among all lanthanides, the visible emitters Tb^{III} and Eu^{III} can be effectively sensitized by UV-absorbing chromophores.[13c] With the natural sensitizers in proteins being UV absorbers, we selected Tb^{III} to prepare "lanthano" Anx V. A single tryptophan and 12 tyrosines would provide the aromatic residues in Anx V for Ln sensitization.[14] The average dissociation constant for Tb^{III} binding to Anx V had been previously reported to be 0.2 μM, and we further confirmed that binding saturation was observed with a 10:1 Tb^{III}-to-protein ratio (Figure S6 in the SI).[14b] We therefore treated purified Anx V with 10 equiv $TbCl_3$ for reconstitution and confirmed its structural similarity to Ca^{II}-Anx V via circular dichroism (Figure S4 in the SI).

Ln reconstitution in Anx V was clearly depicted by the presence of emission bands at 490, 544, and 584 nm, which were characteristic of Tb^{III} emission (Figure 2a). The excitation

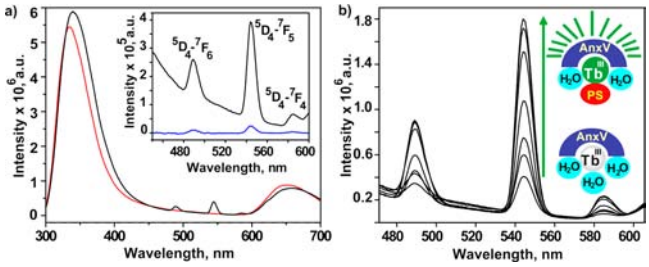

Figure 2. (a) Emission spectrum of 2.8 μM Tb^{III}-AnxV (black) and Anx V (red), pH 6.8, 25 °C. Inset: Tb emission from Tb^{III}-AnxV (black) and $TbCl_3$ (blue). (b) Luminescence response of Tb^{III}-AnxV with PS (0−70 μM).

wavelength was chosen at 283 nm by scanning the excitation spectrum of Tb^{III}-Anx V at 544 nm (Figure S5 in the SI). The protein aromatic amino acid emission peak at 334 nm shifted to 340−342 nm upon Tb^{III}-Anx V reconstitution (Figure 2a). Direct excitation of a $TbCl_3$ solution at 283 nm was used as a comparison to further confirm reconstitution. The extremely low emission intensities of the $TbCl_3$ solution, as shown in Figure 2a (inset), indicated that free Tb^{III} would barely have any contribution to the observed Tb^{III}-Anx V emission.

The response of Tb^{III}-Anx V to PLs was examined using unilamellar PL vesicles, which serve as cell membrane mimics. PS, phosphatidylcholine (PC), asolectin (AL), and mixed PS−PC vesicles were prepared (see the SI). Upon the addition of PS vesicles to reconstituted Tb^{III}-Anx V, we observed a significant increase (6 times at 544 nm) in the emission intensities of the Tb^{III} peaks (Figure 2b). A control experiment with $TbCl_3$, in the presence of PS, showed no increase in the emission intensity, proving that the observed response was due to the direct interaction of PS with the "lanthano" protein (Figure S8a in the SI).

We then compared the emission intensities in the presence of other PLs (Figure 3a). PC accounts for a major 40−50% of the

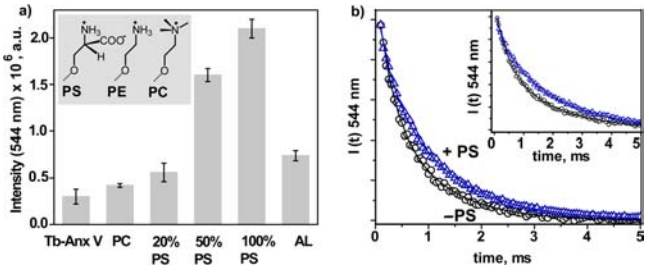

Figure 3. (a) Luminescence response of Tb^{III}-AnxV with PLs. (50 μM). (b) Lifetime measurements for Tb^{III}-AnxV in H_2O (inset: D_2O) without PS (black) and with PS (50 μM) (blue).

total PLs present on the cell membrane[1b] and hence is an obvious choice for checking the specificity toward PS. Unlike PS, the PC vesicles did not elicit any increase in emission upon interaction with Tb^{III}-Anx V (Figure 3a and Figure S10 in the SI). AL is a mixture of other membrane PLs including PC, phosphatidylethanolamine (PE), and phosphatidylinositol but excluding PS. AL showed a 2.5 times increase in the emission intensity with Tb^{III}-Anx V. PE, a major component of AL, has previously been reported to have an affinity toward Anx V.[7d,9] Hence, the observed increase indicates that "lanthano" Anx V effectively mimics the binding specificities of the protein. The headgroup structures of PS, PE, and PC are shown in Figure 3a. The PE headgroup differs from the PS headgroup because it lacks the carboxylate group. The amine moiety of the PE headgroup might still interact with the negatively charged residues in the binding domain.[7d] However, the bulky PC headgroup does not interact with the protein, and hence we observe a distinct selectivity for PS over PC. To further validate our results, we prepared mixed vesicles of PS and PC with increasing PS content. The Tb^{III}-Anx V emission intensity increased as predicted with increasing PS (Figure 3a). These results directly demonstrated the utility of the reconstituted protein toward "turn-on" sensing of PS.

In order to test the response of the "lanthano" protein toward other biologically relevant phospho anions, we measured the emission intensities of Tb^{III}-Anx V with ATP and KH_2PO_4. Neither species afforded an "on" response in the presence of the lanthano Anx V (Figure S8b in the SI). The emission response of Tb^{III}-Anx V to PS was found to be unaltered within a pH range of 5−8 (Figure S11 in the SI). Importantly, Tb^{III}-Anx V turned on upon binding to PS even in the presence of Ca^{II} (Figure S9 in the SI) and also chloride and acetate containing buffers, indicating that these essential ions do not interfere with the sensing scheme.

The PS sensing results are in excellent agreement with our hypothesis that Tb^{III}-Anx V would report on the PS binding event accurately and that the displacement of water molecules on Ln^{III} by PS would cause the sensor to "turn-on". A direct proof for the "turn-on" sensing mechanism was obtained via Ln luminescence lifetime measurements (Figure 3b). The number of inner-sphere water molecules on a lanthanide is quantifiable via a modified Horrocks equation (see the SI), which correlates the number of coordinated water molecules (q) to the Ln

dx.doi.org/10.1021/ic4018932 | Inorg. Chem. 2013, 52, 12314−12316

luminescence lifetimes.[15] Lifetime measurements for Tb^{III}-Anx V in the absence and presence of PS were performed in water and D_2O, respectively (Figure 3b). The lifetime values in water increased upon the addition of PS. A similar increase was also observed in D_2O. Increased lifetimes upon PS addition directly report on the replacement of water molecules from the inner sphere of Tb^{III} which leads to reduced quenching of terbium luminescence. The lifetime value remained essentially unchanged upon PC addition (Figure S12a,b in the SI), demonstrating once again the PS-specific response of Tb^{III}-Anx V.

The modified Horrocks equation was used to calculate the q values before and after PS binding (Table 1). The q value for

Table 1. Lifetime Data for Tb^{III}-Anx V and Calculated q Values

sample[a]	τ_{H_2O} (ms)[b]	τ_{D_2O} (ms)[b]	q
Tb^{III}-Anx V	0.65 ± 0.02	1.20 ± 0.01	3.2 ± 0.2
Tb^{III}-Anx V + PS	0.87 ± 0.01	1.51 ± 0.08	2.1 ± 0.2
Tb^{III}-Anx V + PC	0.68 ± 0.02	1.21 ± 0.07	2.9 ± 0.3

[a]pH 6.8. [b]τ_{H_2O} and τ_{D_2O} are lifetime values in H_2O and D_2O.

Tb^{III}-Anx V decreased upon the addition of PS, while the value essentially remained the same for PC addition. Our results indicate that there are 3 water molecules per Tb^{III} site on an average in Tb^{III}-Anx V, which decreases to an average of 2 upon PS binding. Anx V has several calcium binding sites out of which the AB and B sites have been mainly implicated in PS binding (Figure 1b).[7c,e] According to previous PS docking studies on the three Ca^{II} sites in domain I, the average number of water molecules before PS binding is 3 and drops down to 1.3 upon PS binding.[16] Specifically, the modeling studies indicated that 2 water molecules on the AB site are displaced by 2 PS molecules, while the other sites have more water molecules left after PS binding. Because Ln^{III} ions are established mimics of Ca^{II}, our results prove that PS binding in Anx V causes the displacement of coordinated water molecules. This study is, to our knowledge, the first report providing solution-based molecular evidence on the displacement of metal-ion-coordinated water molecules in human Anx V by PS and distinctly validates our "turn-on" sensing mechanism.

In conclusion, we have demonstrated a simple, yet specific "turn-on" detection strategy for PS, by using Tb^{III}-Anx V. The "lanthano" protein detection system is based on a molecular understanding of the specificity of Anx V for anionic PLs and probes the exact metal ion−PL binding event. A great advantage of Ln-based systems is that the emission wavelengths can be anywhere from the visible to the near-infrared depending on the Ln used. We have demonstrated the proof-of-concept using Tb^{III}; however, other lanthanides will be equally applicable, provided appropriate aromatic antennas are engineered onto the protein. Finally, we believe that this "turn-on" sensing strategy will be applicable to other Ca^{II}-binding peripheral proteins and will help to illuminate crucial metalloprotein-membrane interactions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ankona@tifr.res.in.

Present Address

‡Max Planck Institute of Molecular Physiology, Germany.

Author Contributions

†S.G. and S.M. contributed equally to the work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

A.D. acknowledges support from the Department of Atomic Energy, India, and a start-up research grant from TIFR, India.

■ REFERENCES

(1) (a) Shevchenko, A.; Simons, K. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 593. (b) Leventis, P. A.; Grinstein, S. In *Annual Review of Biophysics*; Rees, D. C., Dill, K. A., Williamson, J. R., Eds.; Annual Reviews Inc.: Palo Alto, CA, 2010; Vol. *39*, p 407.

(2) Lizarbe, M. A.; Barrasa, J. I.; Olmo, N.; Gavilanes, F.; Turnay, J. *Int. J. Mol. Sci.* **2013**, *14*, 2652.

(3) (a) Demchenko, A. P. *Cytotechnology* **2013**, *65*, 157. (b) Fairn, G. D.; Schieber, N. L.; Ariotti, N.; Murphy, S.; Kuerschner, L.; Webb, R. I.; Grinstein, S.; Parton, R. G. *J. Cell Biol.* **2011**, *194*, 257. (c) Schutters, K.; Reutelingsperger, C. *Apoptosis* **2010**, *15*, 1072.

(4) Zheng, H.; Wang, F.; Wang, Q.; Gao, J. M. *J. Am. Chem. Soc.* **2011**, *133*, 15280.

(5) Schoenberger, J.; Bauer, J.; Moosbauer, J.; Eilles, C.; Grimm, D. *Curr. Med. Chem.* **2008**, *15*, 187.

(6) (a) Yoon, Y.; Lee, P. J.; Kurilova, S.; Cho, W. *Nat. Chem.* **2011**, *3*, 868. (b) Smith, B. A.; Akers, W. J.; Leevy, W. M.; Lampkins, A. J.; Xiao, S. Z.; Wolter, W.; Suckow, M. A.; Achilefu, S.; Smith, B. D. *J. Am. Chem. Soc.* **2010**, *132*, 67. (c) Tait, J. F.; Smith, C.; Blankenberg, F. G. *J. Nucl. Med.* **2005**, *46*, 807. (d) Surman, A. J.; Kenny, G. D.; Kumar, D. K.; Bell, J. D.; Casey, D. R.; Vilar, R. *Chem. Commun.* **2011**, *47*, 10245. (e) Quinti, L.; Weissleder, R.; Tung, C. H. *Nano Lett.* **2006**, *6*, 488.

(7) (a) Gerke, V.; Creutz, C. E.; Moss, S. E. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 449. (b) Verdaguer, N.; Corbalan-Garcia, S.; Ochoa, W. F.; Fita, I.; Gomez-Fernandez, J. C. *Embo J.* **1999**, *18*, 6329. (c) Jin, M.; Smith, C.; Hsieh, H. Y.; Gibson, D. F.; Tait, J. F. *J. Biol. Chem.* **2004**, *279*, 40351. (d) Swairjo, M. A.; Concha, N. O.; Kaetzel, M. A.; Dedman, J. R.; Seaton, B. A. *Nat. Struct. Biol.* **1995**, *2*, 968. (e) Montaville, P.; Neumann, J. M.; Russo-Marie, F.; Ochsenbein, F.; Sanson, A. *J. Biol. Chem.* **2002**, *277*, 24684.

(8) Tait, J. F. *J. Nucl. Med.* **2008**, *49*, 1573.

(9) Blackwood, R. A.; Ernst, J. D. *Biochem. J.* **1990**, *266*, 195.

(10) Huber, R.; Berendes, R.; Burger, A.; Schneider, M.; Karshikov, A.; Luecke, H.; Romisch, J.; Paques, E. *J. Mol. Biol.* **1992**, *223*, 683.

(11) Jeppesen, B.; Smith, C.; Gibson, D. F.; Tait, J. F. *J. Biol. Chem.* **2008**, *283*, 6126.

(12) Meers, P.; Mealy, T. *Biochemistry* **1993**, *32*, 11711.

(13) (a) Wartenberg, N.; Raccurt, O.; Bourgeat-Lami, E.; Imbert, D.; Mazzanti, M. *Chem.—Eur. J.* **2013**, *19*, 3477. (b) de Bettencourt-Dias, A.; Barber, P. S.; Bauer, S. *J. Am. Chem. Soc.* **2012**, *134*, 6987. (c) Dossing, A. *Eur. J. Inorg. Chem.* **2005**, 1425. (d) Bunzli, J. C. G.; Eliseeva, S. V. *Chem. Sci.* **2013**, *4*, 1939. (e) Butler, S. J.; Parker, D. *Chem. Soc. Rev.* **2013**, *42*, 1652. (f) Andolina, C. M.; Morrow, J. R. *Eur. J. Inorg. Chem.* **2011**, 154. (g) Zhang, J.; Shade, C. M.; Chengelis, D. A.; Petoud, S. *J. Am. Chem. Soc.* **2007**, *129*, 14834.

(14) (a) Almeida, P. F. F.; Sohma, H.; Rasch, K. A.; Wieser, C. M.; Hinderliter, A. *Biochemistry* **2005**, *44*, 10905. (b) Funakoshi, T.; Furushima, F.; Mizokami, H.; Kojima, S. *Biochem. Mol. Biol. Int.* **1996**, *38*, 965.

(15) Beeby, A.; et al. *J. Chem. Soc., Perkin Trans. 2* **1999**, 493.

(16) Merzel, F.; Hodoscek, M.; Janezic, D.; Sanson, A. *J. Comput. Chem.* **2006**, *27*, 446.