We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



3

The Use of Reductive Methylation of Lysine Residues to Study Protein-Protein Interactions in High Molecular Weight Complexes by Solution NMR

Youngshim Lee[1], Sherwin J. Abraham[2] and Vadim Gaponenko[1,*]
[1]*Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL*
[2]*Department of Molecular and Cellular Physiology, Stanford University, School of Medicine, Beckman Center, Stanford, CA USA*

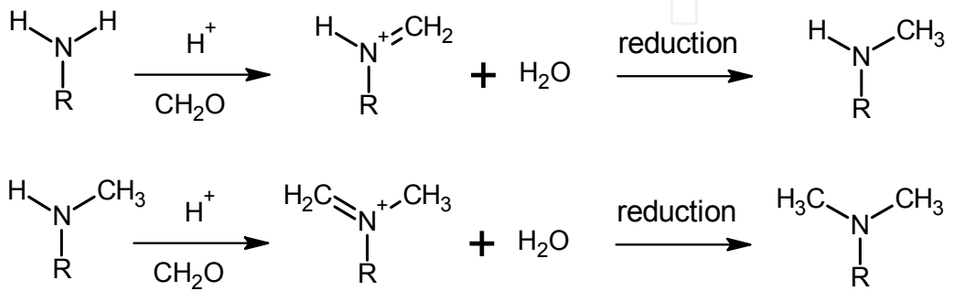
1. Introduction

While solution state NMR is very well suited for analysis of protein-protein interactions occurring with a wide range of affinities, it suffers from one significant weakness, known as the molecular weight limitation. This limitation stems from the efficient nuclear relaxation processes in macromolecules larger than 30 kDa (Wider & W**ü**thrich, 1999). These relaxation processes cause rapid decay of NMR signals. Although the use of transverse relaxation optimized spectroscopy (TROSY) approaches has made solution state NMR of large proteins and protein-protein complexes more feasible, it is still limited by the ability to produce isotope enriched proteins (Pervushin et al., 1997). However, there is a significant number of proteins for which no convenient system for stable isotope incorporation exists. We recently utilized reductive methylation methodology to demonstrate that it is possible to introduce ^{13}C-enriched methyl groups into lysine residues in otherwise unlabeled proteins with the purpose of studying protein-ligand and protein-protein interactions by NMR (Abraham et al., 2008).

Reductive methylation is commonly used to improve crystallization of proteins (Schubot & Waugh, 2004). Studies show that success of protein crystallization improves significantly through reductive methylation of solvent exposed lysines due to a reduction in surface entropy. Reductive methylation does not alter significantly protein structures and native protein-protein interactions (Gerken et al., 1982; Kurinov et al., 2000; Rayment, 1997; Walter et al., 2006). Despite clear advantages offered by reductive methylation, this technique remains underutilized in solution NMR. Here we show that reductive methylation allows characterization of high molecular weight protein-protein complexes that is not achievable using traditional NMR approaches.

* Corresponding Author

For reductive methylation of NMR protein samples, ^{13}C-enriched carbonyl compound (e.g. ^{13}C-formaldehyde) and reducing agents are required. The primary amine of lysine in polypeptide molecules acting as a nucleophile attacks the carbonyl group of formaldehyde. This reaction results in formation of an intermediate imine through the carbonyl-condensation process. The intermediate imine subsequently reacts with a proton donor to give rise to the higher order amine (Scheme 1). The solvent exposed lysine residues are frequently dimethylated when a sufficient amount of formaldehyde is present.



Scheme 1.

The reductive methylation technique offers several advantages. First, proteins purified from their native hosts can be directly used for enrichment with stable isotopes. In this way, the protein molecules are likely to retain their correct fold and post-translational modifications. Second, since only a small amount of ^{13}C-labeled formaldehyde is used in the reaction the reductive methylation procedure is significantly more economical than the traditional isotope enrichment protocols. Finally, the use of ^{13}C-labeled methyl groups in lysines offers an opportunity to observe NMR signals with favorable relaxation properties in large molecular weight proteins due to reduced order parameters for lysine side-chains (Abraham et al., 2009). In this report we not only demonstrate that observation of NMR signals in high molecular weight non-isotope enriched proteins is possible but also that investigation of conformational changes due to binding in protein-protein complexes is amenable to solution state NMR through reductive methylation.

2. Cardiac muscle proteins: Actin, tropomyosin, and troponin complex

Muscle contraction is caused by cyclic interaction between myosin and actin filaments. In cardiac muscle, regulation of contraction is controlled by the troponin complex and tropomyosin which bind to the actin filament (Galińska-Rakoczy et al., 2008; Kobayashi et al., 2008; Kobayashi & Solaro, 2005). The actin filaments consist of polymerized actin (F-actin) molecules which contain myosin binding sites. At rest, the myosin binding site is concealed by tropomyosin forming a coiled-coil dimer that lies in the two grooves of actin. Seven actin molecules interact with one tropomyosin dimer. Each tropomyosin dimer also

binds one troponin complex composed of three subunits: troponin C, troponin I, and troponin T. The N-terminal domain of troponin C has a calcium binding pocket. The troponin complex, together with tropomyosin, regulate muscle contraction in a Ca^{2+}-dependent manner. This is accomplished by altering accessibility of actin binding sites to myosin. Being a Ca^{2+} sensor, troponin functions as an on/off switch for muscle contraction. Muscle contraction occurs when Ca^{2+} binds to the regulatory site in troponin C. Conversely, the muscle relaxes when Ca^{2+} dissociates. When Ca^{2+} concentration is high, Ca^{2+} binding to troponin C induces a structural change in the troponin complex that causes relocation of tropomyosin away from the actin groove. Due to tropomyosin relocation, the myosin binding site on actin is exposed and cross-bridge formation is initiated between actin and myosin. Troponin I is known to inhibit myosin cross-bridge formation by inducing relocation of tropomyosin. Troponin T associates with troponin C and I to form the complete troponin complex. Troponin T also binds to tropomyosin and actin to inhibit myosin binding to thin filaments.

Alpha-helical coiled-coil tropomyosin assembles into filaments in the end-to-end configuration and interacts with actin polymers. When bound to polymerized actin, tropomyosin filament spans seven consecutive actin monomers forming a 369 kDa complex. One troponin binds to each tropomyosin coiled-coil dimer such that the molecular ratio for actin, Tm, and troponin is 7:2:1. There are two kinds of interaction between the troponin complex and actin-tropomyosin. One is Ca^{2+}-independent binding through troponin T, anchoring the troponin complex to actin-tropomyosin. The other is Ca^{2+}-dependent regulatory interactions through inhibitory C-terminal half of troponin I, turning muscle contraction "on" and "off". The cytoplasmic Ca^{2+} concentration is essential for muscle contraction. However, allosteric regulation of the troponin complex is also known to be an important contributor. Solution NMR can detect conformational changes in protein molecules and thus is a good tool to study the allosteric regulation. We utilize the reductive methylation technique because the thin fiber is a large protein-protein complex containing molecules that are difficult to produce as recombinant proteins for enrichment with stable isotopes.

2.1 Conformation of reductively methylated cardiac troponin C free and as part of the cardiac troponin complex in the presence and absence of Ca^{2+}

2.1.1 Methods of preparation of reductively methylated troponin complex

Reductive methylation of troponin C for NMR experiments was performed using ^{13}C-enriched formaldehyde and borane-ammonia complex ($NH_3.BH_3$) as a reducing agent. Briefly, 20 µL of 1 M borane-ammonia complex and 40 µL of ^{13}C formaldehyde were added to 1 mL of troponin C in methylation buffer (10 mM HEPES pH 7.6, 50 mM $MgCl_2$, 50 mM $CaCl_2$, and 1 mM β-mercaptoethanol). The reaction mix was incubated at 4 °C with stirring for 2 h. The procedure was repeated one more time with a final addition of 10 µL of ^{13}C formaldehyde and was incubated at 4 °C with stirring overnight. The reaction was stopped by adding 200 mM glycine and the undesired reaction products and excess reagents were removed by extensive dialysis against 10 mM Tris/HCl pH 7.6, 50 mM $MgCl_2$, 50 mM $CaCl_2$, and 1 mM β-mercaptoethanol. To obtain the troponin complex, troponin I and

troponin T were added to methylated troponin C in the 1:1:1 molar ratio. To obtain larger molecular weight complexes tropomyosin was added to the troponin complex containing methylated troponin C in the 2:1 molar ratio.

The NMR experiments were performed on samples containing 20 μM troponin C (either alone or in complex) in NMR buffer containing 40 mM Tris-HCl (pH 10.0), 50 mM KCl, 1 mM β-mercaptoethanol, and either 50 mM $CaCl_2$ or 50 mM $MgCl_2$. All ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) spectra were acquired on the 600 MHz Bruker Avance spectrometer fitted with a cryoprobe using 128 indirect points at 25 °C. The data were processed using NMRPipe software (Delaglio et al., 1995).

2.1.2 Results

NMR ^{1}H-^{13}C HSQC experiments were performed on free reductively methylated troponin C in the presence and absence of Ca^{2+} and on the cardiac troponin complex containing reductively methylated troponin C in the presence and absence of Ca^{2+}. The results of these experiments are shown in Figure 1. All of the acquired spectra display the expected 12 signals representing methyl groups on eleven lysines in troponin C and one on the N-terminal

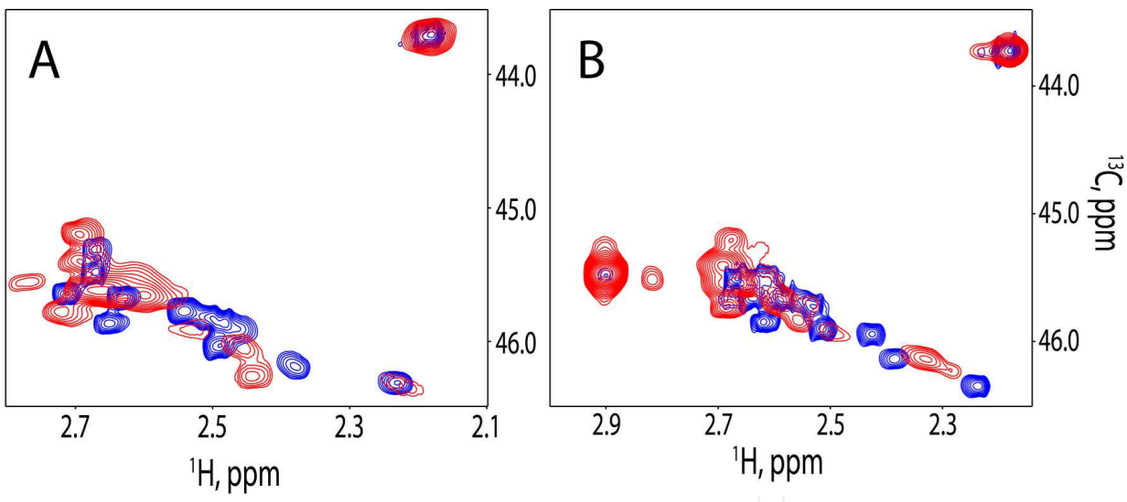


Fig. 1. An overlay wof ^{13}C-^{1}H HSQC spectra of reductively methylated 20 μM troponin C (blue) and the troponin complex consisting of full length troponin C, troponin I, and troponin T (red). The spectra in (A) were recorded in the presence of 50 mM Ca^{2+}. The spectra in (B) were recorded in the absence of Ca^{2+} and the presence of 50 mM Mg^{2+}. The spectra were acquired at 600 MHz at 25 ˚C with 256 indirect points. The buffer conditions are 40 mM Tris-HCl (pH 10.0), 50 mM KCl, 1 mM β-mercaptoethanol, 50 mM $CaCl_2$ (A) or 50 mM $MgCl_2$ (B).

primary amine. Comparison of spectra of free troponin C in the presence and absence of Ca^{2+} (Fig. 1A and 1B) reveals significant differences in methyl chemical shift values. These chemical shift perturbations indicate expected structural rearrangements in the N-terminal domain of troponin C caused by Ca^{2+} binding. Comparison of NMR spectra of free Ca^{2+}-bound troponin C with Ca^{2+}-bound troponin C in the troponin complex reveals significant perturbations in nine out of twelve methyl chemical shifts (Fig. 1A). This observation suggests involvement of troponin C in intermolecular interactions with components of the troponin complex. In the absence of Ca^{2+} only five out of twelve signals experience significant chemical shift perturbations (Fig. 1B). One possible explanation of this is that in the absence of Ca^{2+}, troponin C is less extensively engaged in protein-protein interactions within the troponin complex. Together, we demonstrate that using reductive methylation it is possible to characterize protein-protein interactions within the troponin complex by NMR despite the high molecular weight of the protein system.

2.2 NMR experiments with reductively methylated 369kDa actin-tropomyosin complex suggest that a global conformational rearrangement is induced in polymerized actin upon tropomyosin binding

2.2.1 Methods of preparation of reductively methylated actin-tropomyosin complex

Globular actin was dialyzed into 10mM phosphate buffered saline, pH 7.4, 0.1mM $MgCl_2$, 1mM dithiothreitol, 0.1mM ATP, and 0.01% NaN_3, to make actin filaments. Initially 20mM borane ammonia complex and 40mM ^{13}C-formaldehyde (20% w/w in H_2O) were added into 0.7mL of 60 μM F-actin and the mixture was stirred for 2 hours at 4°C. Addition of borane ammonia complex and ^{13}C-formaldehyde was repeated and mixture was incubated for another 2 hours at 4°C. After incubation, 10 mM borane ammonia complex was added to the mixture. The mixture was incubated at 4°C with stirring overnight. To quench the reaction, the 50 μL of 2M Tris-HCl was added. To study the change in actin structures upon binding of Tm, Tm is added into ^{13}C methylated F-actin to make 7.5 μM of final Tm concentration whereas the concentration of F-actin is 37μM. The molar ratio of actin and tropomyosin was 5 to 1. The samples were dialyzed against 10mM phosphate buffered saline, pH 7.4, with 1mM $MgCl_2$, 0.1mM ATP, 0.01% NaN_3 and 10% D_2O was added for further NMR experiments. All NMR experminents were carried out on Bruker Avance 600 or 900 NMR spectrometers equipped with cryogenic probes. The 2D ^{1}H-^{13}C edited HSQC experiments were processed with NMRPipe software (Delaglio et al., 1995).

2.2.2 Results

To assess conformational changes occurring in polymerized actin upon binding tropomyosin we performed a reductive methylation reaction on actin and carried out ^{1}H-^{13}C HSQC experiments on actin alone and on actin in the presence of tropomyosin (Fig. 2). In the spectrum of polymerized actin seven out of nineteen expected signals were observable. Significant chemical shift changes in four out of seven signals in actin were detected upon Tm binding. Lysines are evenly distributed in the actin structure with no accumulation in any one particular area. Therefore, the data shown here indicates that binding of tropomyosin causes a global conformational change in the structure of polymerized actin.

This observation is contrary to many computational models that propose that tropomyosin binding sites in actin are small and global changes do not occur in the actin-tropomyosin complex.

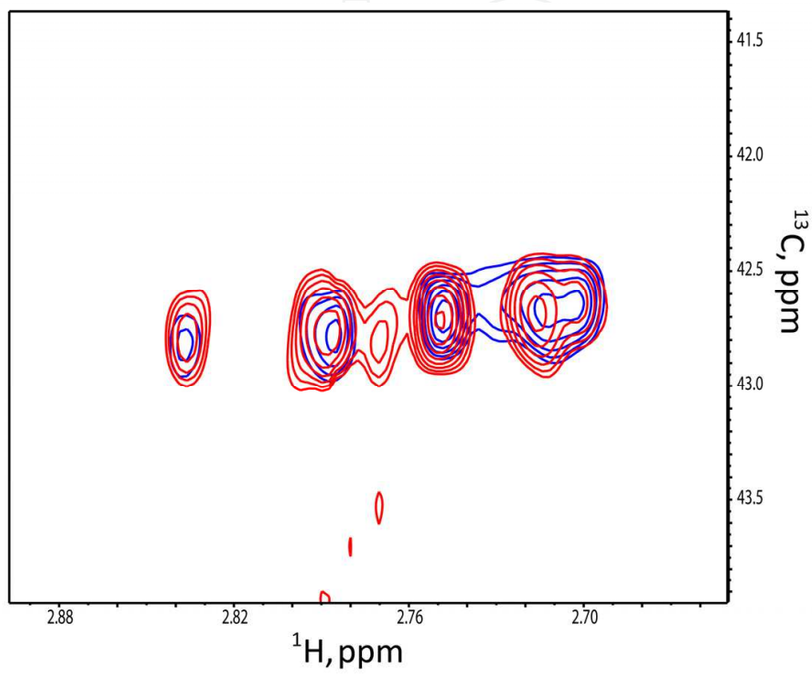


Fig. 2. An overlay of ^{1}H-^{13}C HSQC spectra of reductively methylated polymerized actin (red) and actin-tropomyosin complex (blue). The spectra were acquired at 900 MHz at 25 ˚C with 256 indirect points. The buffer conditions are 10 mM phosphate buffered saline (pH 7.4), 150 mM KCl, 50 mM $MgCl_2$, and 1 mM ATP.

3. Conclusion

In conclusion, we have described an important novel application of the reductive methylation methodology to observation of conformational changes in high molecular weight protein-protein complexes by NMR. Using cardiac troponin C as a model system, for which structural information is available, we confirmed that the proposed methodology allows detection of conformational rearrangements in cardiac troponin C upon Ca^{2+} binding. This was done in the context of the full-length troponin complex. Similar experiments would have been very difficult to perform using conventional NMR approaches due to the high molecular weight limitation. We also show that reductive methylation can be used to discover novel conformational changes in a 369 kDa actin-tropomyosin complex. For the first time we show that actin undergoes a global conformational change upon tropomyosin binding. This appears to be the only way such molecular events can be observed. The available computational models were unable to

predict this phenomenon. Electron microscopy images of the cardiac thin fiber are too low resolution to detect a conformational change in actin. Crystallization of polymerized actin is not feasible due to heterogeneity of actin fibers. In addition, there is no good procedure for production of recombinant actin that would allow traditional approaches for stable isotope enrichment for NMR. The functional significance of actin conformational rearrangements upon binding of tropomyosin is still under investigation. However, the discovery that these conformational changes occur in actin is a significant step forward.

4. Acknowledgment

We acknowledge Dr. Tomoyoshi Kobayashi at the University of Illinois at Chicago for providing purified actin, tropomyosin, and protein samples for the troponin complex. We also acknowledge support from the National Cancer Institute grant R01CA135341 to Vadim Gaponenko.

5. References

Abraham, S. J.; Hoheisel, S. & Gaponenko, V. (2008). Detection of protein-ligand interaction by NMR using reductive methylation of lysine residues. *J. Biomol. NMR*, Vol. 42, No. 2, pp. 143-148.

Abraham, S. J.; Kobayashi, T.; Solaro, R. J. & Gaponenko, V. (2009). Differences in lysine pKa values may be used to improve NMR signal dispersion in reductively methylated proteins. *J. Biomol. NMR*, Vol. 43, No. 4,, pp. 239-246.

Delaglio F.; Grzesiek S., Vuister G. W., Zhu G., Pfeifer J. & Bax A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR,* Vol. 6, No 3, pp. 277-293.

Galińska-Rakoczy A.; Engel P., Xu C., Jung H., Craig R., Tobacman L. S, & Lehman W. (2008). Structural basis for the regulation of muscle contraction by troponin and tropomyosin. *J. Mol. Biol*., Vol. 379, No. 5, pp. 929–935.

Gerken T. A.; Jentoft J. E., Jentoft N. & Dearborn D. G. (1982) Intramolecular interactions of amino groups in ^{13}C reductively methylated hen egg-white lysozyme. *J. Biol. Chem.*, Vol. 257, No. 6, pp. 2894–2900.

Kobayashi T.; Jin L. & de Tombe P. P. (2008) Cardiac thin filament regulation. *Pflugers Arch.* Vol. 457 No. 1, pp. 37–46.

Kobayashi T. & Solaro R. J. (2005) Calcium, thin filaments and the integrative biology of cardiac contractility. *Annu. Rev. Physiol.* Vol. 67, pp. 39-67.

Kurinov I. V.; Mao C., Irvin J. D. & Uckun F. M. (2000) X-ray crystallographic analysis of pokeweed antiviral protein-II after reductive methylation of lysine residues. *Biochem. Biophys. Res. Commun.*, Vol. 275, No. 2, pp. 549–552.

Pervushin K.; Riek R., Wider G. & Wüthrich K. (1997) Attenuated T_2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc. Natl. Acad. Sci. USA* Vol. 94, No. 23, pp. 12366-12371.

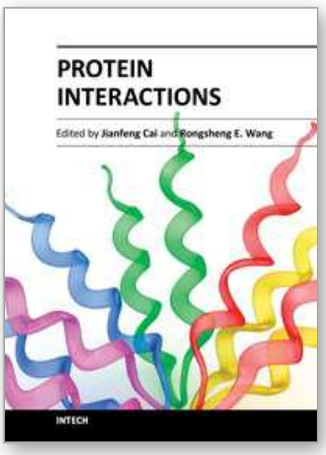
Rayment I. (1997) Reductive alkylation of lysine residues to alter crystallization properties of proteins. *Methods Enzymol.* Vol. 276, pp. 171–179.

Schubot F. D. & Waugh D. S. (2004) A pivotal role for reductive methylation in the *de novo* crystallization of a ternary complex composed of *Yersinia pestis* virulence factors YopN, SycN and YscB. *Acta Crystallogr., Sect D; Biol. Crystallogr.* Vol. 60, pp. 1981-1986.

Walter T. S.; Meier C., Assenberg R., Au K. F., Ren J., Verma A., Nettleship J. E., Owens R. J., Stuart D. I. & Grimes J. M. (2006) Lysine methylation as a routine rescue strategy for protein crystallization. *Structure* Vol. 14, No. 11, pp. 1617–1622.

Wider G. & Wüthrich K. (1999) NMR spectroscopy of large molecules and multimolecular assemblies in solution. *Curr. Opin. Struct. Biol.* Vol. 9, No. 5, pp. 594-601.

IntechOpen



Protein Interactions

Edited by Dr. Jianfeng Cai

ISBN 978-953-51-0244-1
Hard cover, 464 pages
Publisher InTech
Published online 16, March, 2012
Published in print edition March, 2012

Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Youngshim Lee, Sherwin J. Abraham and Vadim Gaponenko (2012). The Use of Reductive Methylation of Lysine Residues to Study Protein-Protein Interactions in High Molecular Weight Complexes by Solution NMR, Protein Interactions, Dr. Jianfeng Cai (Ed.), ISBN: 978-953-51-0244-1, InTech, Available from: http://www.intechopen.com/books/protein-interactions/the-use-of-reductive-methylation-of-lysine-residues-to-study-protein-protein-interactions-in-high-mo

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen