NMR reveals novel mechanisms of protein activity regulation

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Abstract: NMR spectroscopy is one of the most powerful tools for the characterization of biomolecular systems. A unique aspect of NMR is its capacity to provide an integrated insight into both the structure and intrinsic dynamics of biomolecules. In addition, NMR can provide site-resolved information about the conformation entropy of binding, as well as about energetically excited conformational states. Recent advances have enabled the application of NMR for the characterization of supramolecular systems. A summary of mechanisms underpinning protein activity regulation revealed by the application of NMR spectroscopy in a number of biological systems studied in the lab is provided.

Keywords: NMR; protein function; protein regulation; allosteric interactions; autoinhibition

Introduction

The precise regulation of protein activity is essential for the normal functioning of most, if not all, cellular processes. Such regulation is important to prevent premature and undesirable interactions among cellular components and ensure that binding or catalytic activity is switched on only when it is both spatially and temporally appropriate.

Allosteric regulation, which is ubiquitously used in biological processes, is arguably one of the most important mechanisms of protein activity control.^{1–5}

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The cornerstone of this mechanism is the intrinsic capacity of proteins to switch functional states as a response to signal, which is typically triggered by small effector or other protein or nucleic acid binding and posttranslational modifications. Over the recent years, the view and understanding of the fundamental principles underlying allostery have been enriched and often utterly reshaped as techniques such as NMR spectroscopy has been offering insights complementary to those provided by static structures.^{6–11} The early crystallographic work on allosteric systems helped to advance and establish a purely structural, “mechanical” view.¹² Nevertheless, because allostery is fundamentally thermodynamic in nature, long-range communication may be mediated not only by changes in the mean conformation (enthalpic contribution) but also by changes in the dynamic fluctuations about the mean conformation (entropic contribution).^{13,14}

Several proteins have the intrinsic capacity to control their activity through intramolecular

Figure 1. Effect of the anticooperative binding of cAMP to CBD of CAP (CAP^N). Binding of the first cAMP changes the structure of the liganded subunit but has no effect on the mean structure of the unliganded subunit. In contrast, the slow motions (s-ms) of the unliganded subunit are stimulated (denoted by the thicker red line of the cAMP-binding site). Binding of the second cAMP suppresses the fast motions (ps-ns) on both subunits (denoted by the blue color). As a result, binding of the second cAMP incurs an unfavorable conformational entropy change, which is the source of the negative cooperativity (from Ref. 31).

interactions that typically result in negative regulation and form the basis of an autoinhibitory mechanism.^{15–17} Truncations or mutations that disrupt the autoinhibitory interactions often result in a constitutively active functional form wherein no activity control is possible, leading to aberrant function.^{18,19} Autoinhibition is increasingly seen in proteins involved in a diverse set of biological phenomena.^{15–17,20–23} Mechanisms that are known to relieve autoinhibition include posttranslational modification, proteolysis, and addition of proteins or small ligands. The mechanisms and especially the thermodynamics and kinetics of the activation process in autoregulated proteins are generally poorly understood. Since the active site is buried in the inhibited system, ligand binding requires conformational changes that render the site more accessible. Very often, this is accomplished by allosteric effector binding to regulatory sites that are distinct from the binding site. Another potential mechanism is that the ligand binds to a less-populated excited state wherein the binding site is more accessible. Nevertheless, direct evidence of the presence of such excited states is sparse. Dissecting the mechanisms underpinning the activation of autoregulatory systems is very important but remains poorly understood.

NMR spectroscopy, among other techniques, has been instrumental in exploring the different mechanisms used by proteins to regulate their function, activity, and activation. NMR, in addition to providing structural information of a biological system in solution, is unique in providing information about the amplitude of motions taking place on a wide range of time scales,^{24–26} accessing lowly populated conformational states⁷ and providing a residue-specific information of conformational entropy changes upon binding.^{27–29} A summary of mechanisms underpinning protein activity regulation revealed by the application of NMR spectroscopy in a number of biological systems studied in the lab.

Dynamics-Driven Allostery

A fundamental question in allostery is how perturbation at one site is transmitted through the protein to remote sites to effect binding or enzymatic activ-

ity regulation. It is generally thought that changes in protein shape and bonding interactions, which are considered to primarily contribute to enthalpy, are necessary to propagate binding signals to remote sites. Nevertheless, the possibility of allosteric regulation through dynamic (entropic) mechanisms has long been recognized.³⁰ By studying the binding of effector molecules to an allosteric protein, we were able to provide the first experimental evidence that allosteric signal can be propagated through the protein and across subunits with minimal or in the total absence of structural changes.³¹ More specifically, we characterized the binding of cAMP by the catabolite activator protein (CAP), a homodimeric transcription regulator with each subunit consisting of a cAMP-binding domain (CBD) and a DNA-binding domain (DBD).³² Two cAMP molecules bind to dimeric CAP with negative cooperativity and function as allosteric effectors by increasing the protein's affinity for DNA. We exploited the strong negative cooperativity of cAMP binding to CBD of CAP to “freeze” binding conformations at intermediate stages and used NMR and isothermal titration calorimetry (ITC) to characterize them (Fig. 1).³¹

On the basis of chemical shift perturbation, which is a very sensitive measure of changes in the average protein conformation, it was found that binding of the first cAMP to CAP did not induce long-range structural effects to the unliganded subunit. In sharp contrast, the intrinsic motions of CAP residues in the unliganded subunit were strongly affected upon cAMP binding. Thus, it appears that the unliganded subunit “senses” the presence of the ligand (cAMP) in the liganded subunit only through changes in protein motions but not through structural changes. Notably, the data indicated stimulation of fluctuations about the mean structure on the slow (s-ms) timescale, despite the fact that no change in the mean structure was detected.

It is particularly noteworthy that slow and fast motions of residues located at distant regions were affected in the absence of a visible connectivity pathway. This result further undermines the mechanical

view of allostery, wherein binding effects are assumed to propagate through a series of conformational distortions. Rather, the ligand-induced redistribution of the protein's dynamic fluctuations affects regions linked by cooperative interactions, thereby providing a means of propagating the allosteric signal to the distal site even in the absence of structural changes.

The thermodynamic basis of the observed anticooperative binding of cAMP to CAP is entirely of entropic nature.³¹ Estimation of the conformational entropy from changes of order parameters indeed confirmed that the calorimetrically measured difference in entropy between the two sequential binding steps was primarily due to alterations in protein flexibility. The larger conformational entropic penalty in the second step greatly decreases the total entropy of the system, resulting in weaker and, thus, anticooperative binding.

The anticooperative binding of cAMP to CAP cannot be accounted for on the basis of structural changes but can be satisfactorily explained by the experimentally determined changes in flexibility and thus of binding entropy. This could very well be the case for other systems showing negative cooperativity for which crystallographic studies have not revealed any obvious structural effect on the unfilled subunit in the intermediate complex.^{33–35}

Numerous examples are currently available highlighting the inextricable link between protein dynamics and allostery^{8,36}. Long-range allosteric coupling between sites through changes in internal dynamics were seen between the nucleotide-binding cleft and the preprotein-binding site in SecA ATPase,³⁷ between the coordination Na⁺ site and exosite I in thrombin that regulates enzyme's specificity,³⁸ between the substrate-binding site and distal loop in dihydrofolate reductase,³⁹ between the mixed lineage leukemia)- and c-Myb binding sites of the KIX domain of CREB-binding protein,⁴⁰ in PDZ signaling domains,⁴¹ the serine protease inhibitor eglin c,⁴² upon binding of barstar to the RNase barnase,⁴³ in the interaction between the Rho GTPase-binding domain and Rac1,⁴⁴ upon cyclic nucleotide binding to the exchange protein activated by cAMP,^{45,46} a in V-type allosteric enzyme,⁴⁷ and in protein kinase A,^{48,49} only to mention few recent examples from a long list of systems characterized over the years. Dynamic changes in these systems were accompanied by varying extent of structural changes, ranging from minimal to substantial. Local (un)folding transitions and intrinsic disorder have also been shown to underpin allostery in several systems.^{37,50–52}

Dynamic Activation of Protein Function

It is widely accepted that allosteric proteins exist in different conformational states, typically an active

and an inactive, and binding of the allosteric effector shifts the equilibrium, thereby modulating protein activity. Failure of an effector to induce and stabilize the active conformation is expected to result in suppressed protein activity. Thus, allosteric regulation is thought to be mediated exclusively through structural transitions that select and stabilize appropriate conformational states.

The cAMP-mediated allosteric transition in CAP that activates the protein for DNA binding⁵³ is a characteristic example of the widely held view that allosteric regulation is predominantly structural in origin. The DBD in cAMP-free CAP (apo-CAP) adopts an orientation that is incompatible with DNA binding [Fig. 2(A)]. cAMP binding to the CBD of CAP elicits allosterically a pronounced conformational change to DBD, which undergoes a ~60° rigid-body rotation [Fig. 2(A)]. In this orientation, the recognition helices (F-helices) are optimally poised to interact with the major groove of the DNA and the affinity of CAP for DNA is enhanced by several orders of magnitude.⁵⁴ Therefore, the structural basis for cAMP-induced CAP activation consists of a marked alteration of the relative orientation of the DBDs.

Surprisingly, cAMP binding to CAP-S62F mutant fails to elicit the allosteric transition and DBD remains in the inactive conformation [Fig. 2(B)].⁵⁵ In fact, results from relaxation dispersion experiments suggest that the active conformation is present in CAP-S62F-cAMP₂ but it is marginally stabilized, and, thus, poorly populated (~2%).⁵⁵ Thus, CAP-S62F would be expected to bind to DNA with at least 50-fold lower affinity than WT-CAP. Nevertheless, CAP-S62F-cAMP₂ binds to DNA very tightly with complex formation being entirely driven by a very favorable conformational entropy change as measured by ITC. In sharp contrast, WT-CAP-cAMP₂ binding to DNA is entirely driven by a large enthalpy change as it incurs a very unfavorable entropy change. It is of particular interest that the NMR-measured per-residue conformational entropy changes for the interaction of WT-CAP-cAMP₂ and CAP-S62F-cAMP₂ proteins with DNA shows that whereas the wild type complex is accompanied by a large unfavorable conformational entropy, formation of the mutant complex incurs a very favorable conformational entropy. Taking into account that the contribution of hydration phenomena to CAP-DNA binding should be very similar for both WT-CAP and CAP-S62F proteins, the large entropy difference measured by ITC for the formation of the two DNA complexes can be attributed to the dramatically different conformational entropy change of binding. The collective data provide strong evidence that CAP-S62F-cAMP₂ is “dynamically” activated for DNA binding.

The cAMP-binding module is used by hundreds of proteins to bind to various cofactors and translate

Figure 2. Reaction pathways for cAMP-mediated CAP activation and DNA binding. (A) cAMP binding to WT-CAP elicits the active conformation so that DBD becomes structurally poised to interact favorably with DNA. Complex formation is strongly enthalpically favored and entropically unfavorable. (B) cAMP binding to CAP-S62F stabilizes only marginally the active conformation, which is poorly populated (~2%). Because DNA binds to the active conformation of CAP with many orders of magnitude stronger affinity than to the inactive conformation, DNA will bind selectively to the active, low-populated DBD state and shift the population from the inactive DBD to the active DBD conformation. DNA binding to CAP-S62F-cAMP is entirely driven by entropy, which is dominated by favorable conformational entropy change upon DNA binding (from Ref. 55).

the signal into biological response.^{56–58} In fact, the structural and regulatory adaptability of this scaffold has led to the evolution of an important group of physiologically versatile transcription factors (CAP/FNR family).⁵⁹ It is of particular interest that the present results demonstrate that the dynamics of this module is very adaptable and its distinct response may either drive or suppress, through entropy changes, ligand binding to allosterically coupled remote sites.

These results lend strong support to the emerging view of protein flexibility as a major source of entropy that can influence the thermodynamics of binding. Very interesting results on calmodulin have been particularly revealing about the role of conformational entropy in molecular recognition and allostery.^{60,61} Calmodulin, a central player in calcium-mediated signaling, has been used as a model system to investigate the role of changes in fast (sub-nanosecond) internal dynamics and its associated

conformational entropy in protein–ligand binding. By studying calmodulin in complex with a series of different binding peptides, it was found that the apparent change in conformational entropy was linearly related to the change in the overall binding entropy.⁶⁰ This observation provided strong evidence that changes in protein conformational entropy can contribute significantly to the energetics of protein–ligand association. In fact, empirical calibration of the NMR-derived conformational entropy suggested that the standard approach of summing up the individual contribution of each one of the experimentally accessible bond vectors may in fact underestimate the overall contribution of conformational entropy to the binding free energy.⁶¹ The emerging view of protein flexibility as a major source of conformational entropy that can influence the thermodynamics of binding, also supported by many other examples,^{8,36} indicates that dynamic activation of protein function may be more common than thought.

Protein Activity Regulation by a Proline Switch

Proline *cis*–*trans* isomerization has emerged as a particularly efficient regulatory mechanism in many biological processes, including cell signaling,^{62–65} neurodegeneration,⁶⁶ amyloidogenesis,⁶⁷ channel gating,⁶⁸ gene regulation,^{69,70} phage and virus infection,^{71,72} enzyme function,^{73,74} and ligand recognition.⁷⁵ Proline isomerization is unique in that it exerts its function through multiple mechanisms involving (i) significant conformational changes caused by the 180° rotation about the prolyl bond, (ii) slow kinetics of isomerization affording a molecular timer, and (iii) the recruitment of prolyl *cis*–*trans* isomerase enzymes (PPIases).^{76–79} However, despite the importance of *cis*–*trans* isomerization, there is a surprising paucity of high-resolution structural data elucidating the effect that this process may elicit within a protein. Understanding the mechanisms underlying the function of prolines as molecular switches necessitates the availability of structural data on both the *cis* and *trans* conformers. The limited structural information^{76,77} on both conformers available to date has been suggestive of a rather local effect caused by proline isomerization.

Recent studies^{62,63,78,79} on the Crk signaling protein have shown how proline isomerization yields a functional binary switch. Crk is an important adaptor protein consisting of a single SH2 domain, an N-terminal SH3 (SH3^N) domain, and a C-terminal SH3 domain (SH3^C).⁸⁰ We found that part of the linker tethering the two SH3 domains in Crk interacts with the SH3^C. A single prolyl bond (Gly237–Pro238) undergoes *cis*–*trans* isomerization, thereby regulating the linker–SH3^C interaction giving rise to two distinct conformations. Proline isomerization causes a remarkable reorganization of the interface between the linker and the SH3^C domain, to the extent that the two resultant conformers present drastically different surfaces. The conformational rearrangement induced by *cis*–*trans* isomerization endows the conformers with distinct binding properties: in the *cis* form, the two SH3 domains engage in an intramolecular fashion, giving rise to an autoinhibitory conformation that prevents the binding of physiological ligands, such as the Abl kinase; in contrast, in the *trans* form the two SH3 domains do not interact and Crk exists in a uninhibited, ligand binding–competent conformation (Fig. 3).

Crk provides the first example wherein autoinhibition is controlled by an internal conformational switch afforded by proline isomerization.^{62,63} The results further highlight how a 180° rotation about a single prolyl bond can have such a strong effect on the global structure of the protein. It is only in the *cis* conformer that the SH3^C domain exposes the specific residues that form a binding surface capable of mediating the interaction between SH3^C and SH3^N. In contrast, in the *trans* form, this set of residues is

buried, and thus the SH3^N–SH3^C interaction is not possible. Thus, the proline switch in Crk plays a very important physiological role, as it toggles the protein between a closed and an open conformation. In fact, the open conformation, which is weakly populated (~10%), constitutes the activated form of Crk, in which the polyproline II (PPII)-binding site is accessible to physiological ligands. However, since proline isomerization is intrinsically a very slow process (on the order of minutes),⁷⁸ the kinetics of the molecular switch is particularly slow. It is of particular interest that cyclophilin A (CypA) is recruited and accelerates dramatically the rate of the interconversion to the millisecond regime at physiological temperatures, a more meaningful timescale for modulating the kinetics of biological processes.⁶² Thus, proline isomerization in Crk functions as a molecular timer whereby the energetics and rate of signaling complex formation can be regulated.

Protein Activation via Energetically Excited States

Growing evidence has demonstrated that weakly populated conformational states, which are not readily accessible for characterization, are important for molecular recognition, catalysis, and allostery.^{6,7,25,26,81–84} Relaxation dispersion NMR methods^{24–26} developed over the past decade have enabled detection and characterization of such energetically excited states. The ground and excited states interconvert on the μ s–ms timescale and NMR can provide information about the kinetics and thermodynamics of the exchange process as well as the chemical shifts of the excited state.

Information about such lowly populated conformational states has enabled a better understanding of the activation mechanism of autoinhibited systems, a process that is generally very poorly understood. We have shown that ~10% of the total population of the Crk molecules exist in an open conformation wherein the PPII-binding site on SH3 is completely unoccupied.^{62,63} Each Crk molecule will dynamically sample both conformations. Ligand will bind preferentially to the ~10% of the Crk molecules with accessible SH3^N-binding site, as evidenced by the thermodynamic data, resulting in equilibrium shifting toward the extended, uninhibited conformation. Thus, ligand does not need to induce specific conformational changes in the SH3^N/SH3^C-binding interface, but instead may drive Crk activation by biasing the dynamic conformational ensemble. The kinetics of this process is further modulated by the action of cyclophilin A.⁶²

The autoinhibited DH domain of the Vav proto-oncogene has provided unprecedented insight into the activation process of an autoregulated protein quantitative as well as a correlation between dynamics and function.⁸⁵ The DH domain undergoes excursions to an excited conformation where the autoinhibitory

Figure 3. Mechanistic basis for the regulation of Crk activity by a proline switch. Crk adopts predominantly ($\sim 90\%$) the closed, autoinhibited conformation, but a minor population ($\sim 10\%$) adopts the open, uninhibited conformation in which the PPII-binding site on SH3^N is accessible for binding by ligands such as the Abl kinase. The open conformation exists as an equilibrium between the cis and the trans isomer, but only the cis version forms the closed conformation. The rates of the cis–trans interconversion are regulated by the action of CypA, which accelerates the interconversion by four orders of magnitude (from Ref. 63).

element is dissociated from the catalytic surface. Characterization of a series of mutants with altered population of the excited state showed that catalytic activity is linearly dependent on the population of the excited state.⁸⁵ Characterization of weakly populated states can also provide unique insights into the energy available for allosteric regulation. The kinase activity of the oat phototropin 1 photosensor is regulated by the interaction of a LOV (light, oxygen, and voltage) domain with an adjacent α -helix in a light-dependent manner. The “dark” and “lit” states interconvert on the μs – ms timescale by determining the populations of the two states it was estimated that $\sim 3.8 \text{ kcal mol}^{-1}$ are available for the kinase activation.⁸⁶ In the case of CAP, as described above, NMR and ITC approaches were combined to quantify the energy required ($\sim 2.8 \text{ kcal mol}^{-1}$) for the allosteric transition that switches CAP from its inactive to the active conformation that binds strongly and specifically its cognate DNA site.⁵⁵

Determining the chemical shifts of the excited state using relaxation dispersion have in several cases provided information that those states structurally resemble related functional states.^{40,53,82,83,87} Incorporation of chemical shifts in NMR structure

refinement and the development of new NMR experiments that can report on internuclear vector orientations in excited conformational states provide valuable structural constraints that may enable direct structural characterization of higher energy protein conformations.^{88–90} Kay and coworkers recently reported the structure of an “invisible” protein folding intermediate, a remarkable achievement convincingly showing that structure determination of weakly populated conformational states are within reach.⁹¹ Under favorable conditions, NMR-detected paramagnetic relaxation enhancement (PRE) can be used to determine the structure of weakly populated conformational states.⁹²

Regulation Mechanisms in Large Protein Machineries

Studying by NMR large allosteric systems with multiple-binding sites and intricate functional mechanisms would be truly fascinating. Thanks to the pioneering work from the lab of Lewis Kay it is now possible to obtain both structural and dynamical information on supramolecular protein machineries.^{93–97} The key to achieving this has been the development of a labeling protocol that allows the

Figure 4. Signal sequence binding to SecA. SecA consists of several domains: the nucleotide-binding domain (NBD), the PBD, the intramolecular regulator of ATP hydrolysis 2 domain (IRA2), a long α -helical scaffold domain (SD), the IRA1 hairpin, the winged domain (WD), and the C-tail. (A) The lowest-energy structure of SecA bound to the signal peptide is shown. SecA is displayed as a semi-transparent solvent-accessible surface and the signal peptide is shown in yellow. A ribbon model is displayed below the surface. (B) Closer view of the groove bound to the signal peptide. Green and red surface indicates hydrophobic and acidic residues, respectively. Peptide is shown as a ribbon ball-and-stick representation and most of its residues are numbered. (C–E) The signal sequence binds to a groove formed at the interface between PBD and IRA1. The groove is sterically inhibited by the C-tail and signal sequence binding results in C-tail displacement. SecA exists in an equilibrium between a compact and a more loose conformational state, with the latter being favored by temperature increase and SecEYG binding (from Ref. 101).

specific protonation of methyl groups in an otherwise deuterated background.^{94,98,99} The approach exploits some very favorable properties of methyl groups in proteins: (i) they occur frequently in the hydrophobic cores of proteins, or at the interfaces of biomolecular complexes, and are thus excellent reporters of structure and dynamics; (ii) the three protons of the methyl group all contribute to the intensity of the same signal, and therefore methyl probes are significantly more sensitive than other candidates. Moreover, methyl groups are intrinsically optimized for use in transverse relaxation optimized spectroscopy (TROSY) and the simple ^1H – ^{13}C heteronuclear multiple quantum coherence (HMQC) experiment can be used to select for the pathways with the favorable relaxation properties.¹⁰⁰

By exploiting this approach, we have been able to study by NMR the SecA translocase ATPase.¹⁰¹ SecA is a 204 kDa homodimeric protein consisting of 901 residues per protomer and comprising a number of domains [Fig. 4(A)].¹⁰² The size and complexity of the system rendered assignment of the methyl groups of SecA a particularly daunting task. A do-

main-parsing strategy was followed to assign the methyl groups (Ile, Leu, Met, and Val) with virtually all domains of SecA and a number of fragments comprising contiguous domains being isolated and characterized by NMR.^{37,101} By combining transferred NOESY, line broadening, and PRE experiments, we determined the structure of SecA in complex with a secretory signal peptide [Fig. 4(A,B)]. Recognition of signal sequences by receptors, such as SecA, controls the entry of proteins to export pathways. Until recently, no structure of a signal peptide in complex with its cognate receptor was available. The data showed that while the free peptide is unstructured, its hydrophobic region adopts an α -helical conformation when interacting with SecA. The signal peptide binds into a relatively large groove formed at the interface of two domains. The structure revealed a dual binding mode for the peptide, which is capable of using both its positively charged N-terminus and the α -helical hydrophobic region to interact with the same groove in SecA.

Two additional interesting mechanisms for activity regulation were revealed by the NMR studies

[Fig. 4(C,D)]. First, the C-tail of SecA partially occludes the peptide-binding groove, thereby forming the basis of an autoinhibitory mechanism. Second, the combined NOE and PRE NMR data showed that SecA interconverts between an open (major form, 90%) and a closed conformation (minor form, 10%) in solution, which correspond to the two alternative conformations seen in crystal structures.^{103–105} The large conformational change undergone by the pre-protein-binding domain (PBD) might be a functional one, linking preprotein binding to the catalytic cycle, thus, presenting a simple translocation mechanism.

Conclusions

NMR spectroscopy is a very powerful methodology for the characterization of biomolecular systems. NMR can yield the high-resolution structure of proteins and their complexes, determine the time scale and amplitude of intrinsic protein motions and how they are modified upon binding or during reactions, provide a residue-specific information about the conformation entropy of binding, detect, characterize, and even determine the structure of energetically excited conformational states. Now that the NMR characterization of even supramolecular protein systems is within reach more exciting insight into biological functional mechanisms is expected.

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