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Real-time NMR monitoring of biological activities in complex physiological environments

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Biological reactions occur in a highly organized spatiotemporal context and with kinetics that are modulated by multiple environmental factors. To integrate these variables in our experimental investigations of 'native' biological activities, we require quantitative tools for time-resolved *in situ* analyses in physiologically relevant settings. Here, we outline the use of high-resolution NMR spectroscopy to directly observe biological reactions in complex environments and in real-time. Specifically, we discuss how real-time NMR (RT-NMR) methods have delineated insights into metabolic processes, post-translational protein modifications, activities of cellular GTPases and their regulators, as well as of protein folding events.

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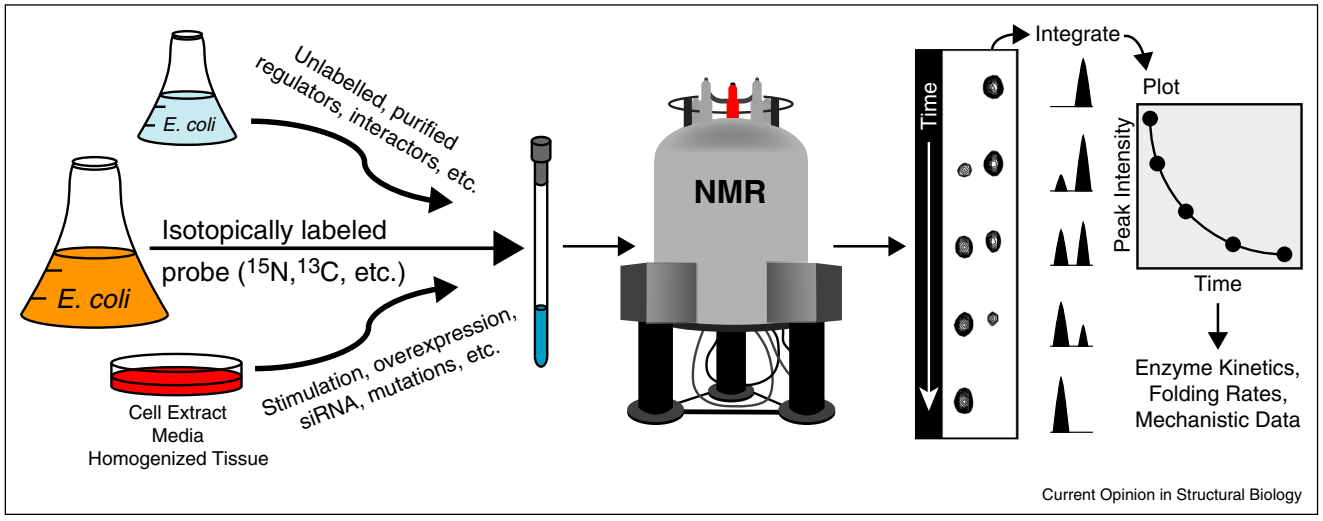
Introduction

Biology is governed by context-dependent, dynamic changes across multiple distance- and time-scales. In turn, an impressive arsenal of biological activities executes and responds to such changes in a highly organized and intricately regulated manner. To study such activities in a meaningful biological context, we require readout tools that allow us to directly assess their structural and functional characteristics in physiological environments. This represents a veritable challenge for most experimental techniques, although coordinated efforts by different disciplines have provided important quantitative and mechanistic insights into a variety of systems [1].

Here, we outline the use of high-resolution, real-time NMR spectroscopy (RT-NMR) to monitor biological activities in a virtually continuous manner, at atomic resolution, and in the complex environments of native cell and membrane extracts, tissue homogenates, biopsy samples, and intact cells. In most of these applications, the biomolecules of interest are labeled with stable isotopes to provide a selective 'visualization filter' that renders the isotope-labeled species the only NMR-visible components in otherwise non-labeled environments. In turn, endogenous biological activities 'act' on the labeled biomolecules, either by directly metabolizing them (exploited in NMR-based metabolic profiling approaches), by chemically modifying them (used in NMR monitoring of cellular post-translational protein modifications), or by modulating the activities of isotope-labeled enzymes via interactions with stimulatory, or inhibitory cellular factors (the basis for studies of cellular enzymes such as small GTPases). One advantage of monitoring reactions in cell extracts is the presence of complete enzymatic and regulatory systems, which can offer comprehensive insight into biological processes. Because these complex environments are difficult to control experimentally, and their compositions only partially known, NMR studies in such environments can be complemented by *in vitro* approaches in reconstituted mixtures of defined compositions and concentrations.

NMR spectroscopy is a non-invasive, non-disruptive and quantitative analytical tool, with NMR signal intensities reflecting the absolute concentrations of isotope-labeled components. Time-dependent changes of these signals provide quantitative information about biological activities acting on NMR-visible components (Figure 1). The 'time-resolution' that is achievable with such RT-NMR approaches depends on the acquisition time of each spectrum in a series of consecutive NMR experiments, which is determined by the type of experiment that is recorded, the concentration and molecular weight of the isotope-labeled species, and the signal-to-noise ratio (SNR) needed to accurately measure individual NMR signal changes (Box 1). In practice, RT-NMR monitoring of metabolic processes is usually performed with consecutive one-dimensional (1D) NMR experiments,

Figure 1



Real-time NMR monitoring of biological activities on proteins. Isotope-labeled proteins are expressed and purified from bacteria, and prepared in a uniform 'starting' state (e.g., non-phosphorylated, co-factor loaded, unfolded, etc.). These NMR-sensitive probes can be combined with various sources of unlabeled biomolecules such as recombinant proteins expressed in bacteria, whole cell extracts or tissue samples, which remain NMR 'invisible'. Successive NMR experiments provide quantitative, temporal monitoring of changes in the labeled probe catalyzed by the components added. The time courses of peak build-up and decay can be used to describe enzyme kinetics or other mechanistic aspects of protein function.

recorded within seconds to minutes. Protein-based RT-NMR studies typically require two-dimensional (2D) NMR experiments, with acquisition times in the range of minutes to hours, and micro-molar (μM) concentrations of isotope-labeled protein samples.

Real-time NMR monitoring of metabolic processes

The underlying rationale of RT-NMR approaches to monitor metabolic processes is to detect the chemical conversions of added, isotope-labeled metabolites via their progressively altered spectral properties, as well as NMR 'signatures' of newly metabolized products. By following these changes as a function of time, signal reductions of original metabolites and the corresponding increases of product NMR signals report on the rates of metabolic processing. In turn, changes in metabolic activities in response to environmental conditions or drug inhibition, for example, lead to differences in the respective rate profiles. Alterations in the chemistry of metabolic processing, as a result of engineered mutations for instance, lead to off-pathway spectral features and unambiguously reveal the presence of alternative metabolic products. This principle of monitoring time-dependent changes of specific NMR signatures serves as the basis for all RT-NMR applications today.

Metabolic RT-NMR has been used to elucidate the complex kinetics of coupled glycolytic enzymes in *Escherichia coli* lysates [2] and of acetylcholine synthesis in hippocampal tissue [3•]. While sensitivity remains a challenge, dynamic nuclear polarization (DNP)-based applications are particularly well suited for metabolite studies and hold great promise for the future [4]. In combination with isotopic labeling, dissolution DNP offers exceptionally high signal enhancements and time-frames for metabolic profiling, because hyperpolarized states of small molecules have long lifetimes. DNP-based RT-NMR applications have been used to analyze disease-associated metabolic changes including glucose metabolism in breast cancer cells [5], 2-hydroxyglutarate production in glioblastoma cells [6], and to study the effects of PI3K or MEK kinase inhibitors on pyruvate metabolism in various cancer cells [7,8]. These techniques are certain to advance the burgeoning field of metabolomics through the added benefit of providing temporal-resolution of metabolic states. Examples of industrial RT-NMR applications include monitoring of metabolic parameters in bioreactors to optimize recombinant protein production [9], observations of xylose processing in bacteria [10], and studies of alcoholic fermentation and glycolysis in yeast [11].

RT-NMR monitoring of post-translational protein modifications

Uniformly, or site-selectively isotope-labeled proteins offer additional possibilities for RT-NMR. Given the unique sensitivity of NMR spectroscopy toward changes in the chemical environment of individual protein residues, biological reactions that target selected amino acids

Box 1 Pulse sequences and methodologies used in RT-NMR.

	Pulse Sequence	Min. Exp't time	Labeling required	Advantages	Disadvantages	Reference
1D	1D ^{1}H NMR	~1 s	None	-High temporal resolution -Sensitivity -No isotopic labelrequired	-Spectral overlap -High background in complex mixtures	*Nat Struct Biol* (1995) 2:865
2D	^{1}H-^{15}N HSQC	~5 min	^{15}N	-Improved peak dispersion -Amides are sensitive reporters of structural changes and PTMs	-2D spectra require longer acquisition time	*Biochemistry* (1996) 35:4306
2D	SOFAST- ^{1}H-^{15}N HMQC	~5 s	^{15}N	-As for ^{1}H-^{15}N HSQC with improved sensitivity and temporal resolution	-Fast pulse programs can impact peak intensity quantification	*PNAS* (2007) 104:11257
2D	^{1}H-^{13}C HMQC	~5 min (less with SOFAST)	Uniform or selective ^{13}C	-Side-chain methyls provide high sensitivity of detection	-Side-chain methyls may be less sensitive to molecular surface events	*J Magn Reson* (1983) 55:301-315
2D	^{1}H-^{1}H TOCSY	Several hours	None	-Probes nucleic acid modifications	-Long acquisition time	*Angew Chem* (2014) 53:2349
3D	BEST HNCO	~10 min (with NUS)	^{15}N and ^{13}C	-Dispersion of peaks in third dimension reduces overlap	-3D spectra require longer acquisition time	*JACS* (2006) 128:9042 *J Biomol NMR* (2014) 58:129
3D	^{1}H-^{15}N HSQC NOESY HSQC	Several hours	^{15}N	-NOEs provide a direct probe of protein folding	-Very long acquisition time	*J Mol Biol* (2003) 328:1161

Sequential Spectra

Experiment time << Reaction time
Spectral 'snapshots' collected sequentially.

Continuous Spectra

Experiment time ~ Reaction time
Kinetics determined by deconvolution of lineshape.
Science (1996) 274:1161

Methods to improve temporal resolution of sequentially collected spectra

Non-Uniform Sampling (NUS)

Reduce number of 2D/3D matrix points to be acquired. Temporal resolution improved several fold by peak-guided data collection.

Fast Pulsing

Shorten time per scan. Temporal resolution improved by reducing delay between scans (e.g., BEST, SOFAST)

Dynamic Nuclear Polarization (DNP)

Enhance sensitivity of detection. Temporal resolution improved by enhancing signal/noise for analyte-of-interest.

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can be followed in a time-resolved and quantitative fashion. To illustrate this, we shall consider a 2D ^{1}H–^{15}N correlation experiment of an ^{15}N isotope-labeled protein, which produces NMR signals for each H–N spin pair in the sample (i.e., backbone and side-chain amides). The chemical environments of protein backbone amides depend on many parameters including the amino acid type, the immediate sequence context, and the secondary/tertiary structure in which they reside. This results in cross-peak positions that are more or less unique. When such an isotope-labeled protein is added to a cell extract containing enzymes that modify individual amino acids, this leads to a decrease of cross-peak intensities of its targeted residues and the concomitant appearance of new NMR signals corresponding to the chemically altered residues. These spectral changes directly identify the modified protein residues and reveal the chemical nature of the respective modifications [12]. By measuring the rates with which these spectral changes occur, time-resolved reaction kinetics can be obtained.

Phosphorylation

Serine, threonine or tyrosine phosphorylation is often observed in physiological environments because kinases and phosphatases are present in large quantities in intact eukaryotic cells, and their enzymatic activities are well preserved in cell extracts [13••]. RT-NMR based applications to monitor protein phosphorylation events have been performed in reconstituted kinase reactions [14], cell extracts [15,16••,17,18] and in intact cells [19,20]. These RT-NMR approaches have the additional advantage of providing mechanistic insights into modification hierarchies, including inhibitory [12] and stimulatory [16••,17,20] 'priming' events in phosphorylation cascades. One benchmark study in this regard is work by Cordier *et al.*, who used RT-NMR to analyze nine consecutive phosphorylation reactions within the intrinsically disordered, C-terminal tail region of the dual lipid–protein phosphatase and tumor suppressor PTEN [16••]. The authors showed that multi-site phosphorylation of two PTEN substrate clusters by CK2 and GSK3β proceeds in a defined order and along an entirely distributive

mechanism, with both kinases establishing consensus and priming-sites for each other along the way (Figure 2a). By delineating these reciprocal activation events from a single set of time-resolved NMR experiments, Cordier *et al.* have laid the groundwork for even more complex studies in this direction. In combination with non-uniform sampling schemes, fast-pulsing acquisitions, and simultaneous spectral processing routines, RT-NMR experiments will likely enable advanced profiling of post-translational protein modification reactions in the future, as exemplified by real-time 3D NMR approaches to monitor tyrosine phosphorylation of CD79b, with a 'time resolution' of less than 10 minutes (i.e. the time requirements for a single NMR spectrum) [21]. The works by Cordier *et al.* and Theillet *et al.* also provide detailed mathematical equations and fitting procedures to derive quantitative kinetic parameters of multi-site phosphorylation reactions from such RT-NMR measurements [13••,16••].

Other PTMs

Another advantage of RT-NMR in profiling post-translational modifications is the ability to jointly delineate residue-resolved trajectories of different types of modifications such as acetylation and methylation [14,22,23]. Because the NMR readout principle simply relies on changes in the chemical environments of modified protein residues, the method is generally applicable to investigate different types of enzyme-mediated and non-enzyme-mediated modifications [12], including monitoring of site-specific oxidation events. Maltsev *et al.* used RT-NMR to study methionine oxidation of the Parkinson's disease-associated protein α-synuclein in the presence of peroxidized lipids [24•]. Their analysis revealed that single site oxidation of Met1, or Met5 initiates independently on individual α-synuclein molecules and proceeds with comparable rates, whereas the build-up of doubly oxidized species ensues after these events (Figure 2b). Fernandez and co-workers obtained similar RT-NMR results in a copper/ascorbate system [25].

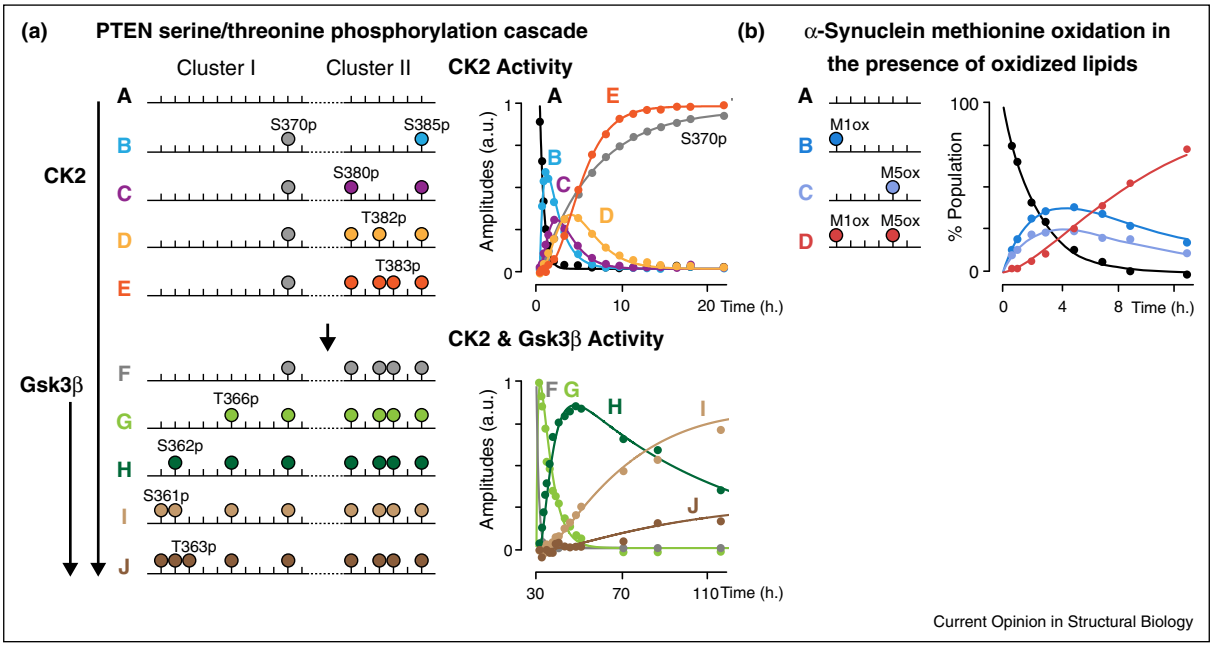
RT-NMR monitoring of protein conformational changes

While RT-NMR approaches can be used to study post-translational modifications on isotope-labeled proteins, they can also be employed to monitor protein conformational exchange, and to derive quantitative information about how different cellular factors modulate the activities of enzymes themselves.

Small GTPase proteins

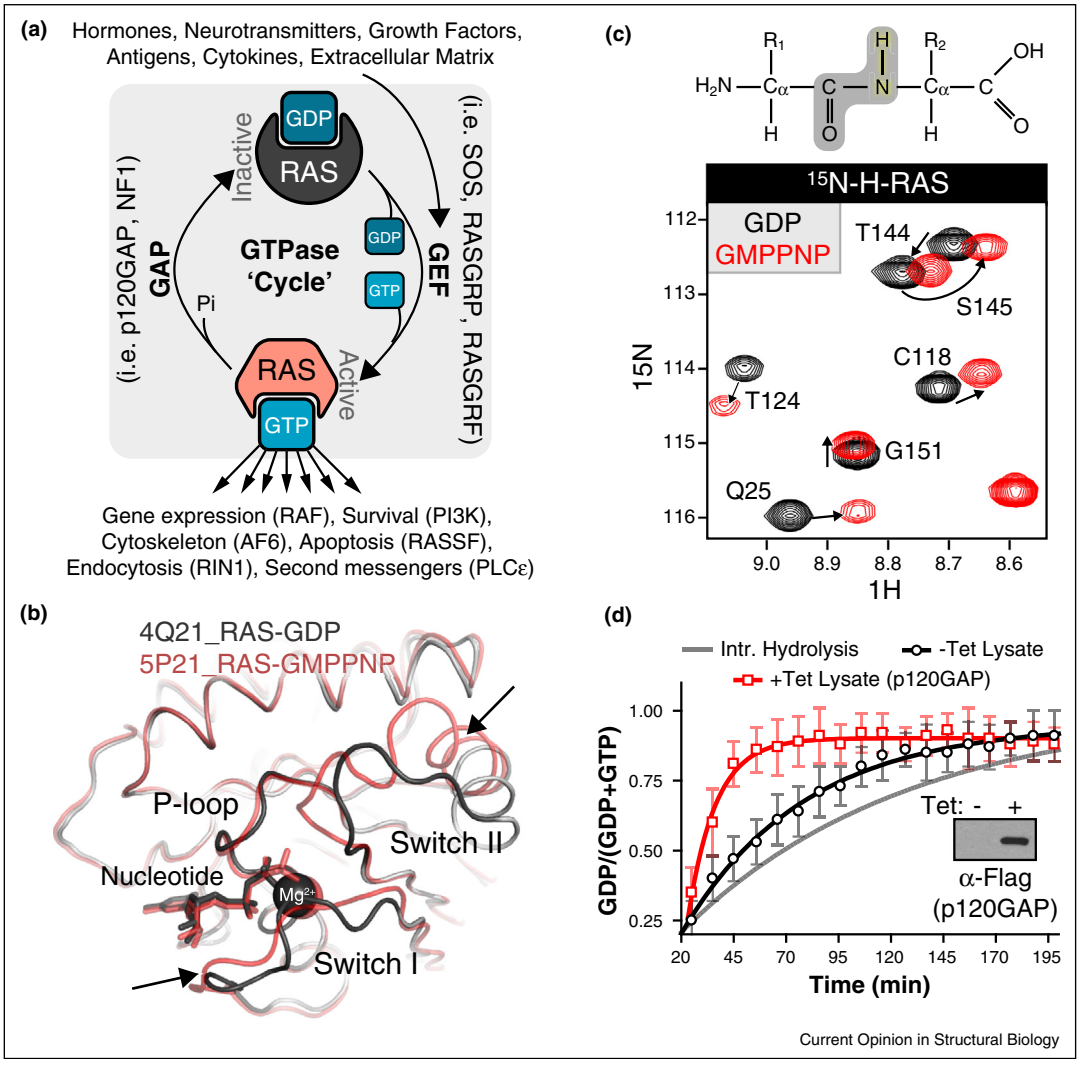
One such RT-NMR application addresses the activities of the Ras-family small GTPases (Figure 3a). GTPases bind and convert GTP to GDP, which involves a substantial structural rearrangement of their catalytic

Figure 2



RT-NMR observations of protein post-translational modifications. **(a)** PTEN phosphorylation reactions by GSK3β and CK2 kinases report on the order and kinetics of clustered multi-site phosphorylation events. Nine sites are modified by CK2 and GSK3β, including two newly identified sites in the PTEN C-terminal tail. The sequence of reactions and their apparent rate constants were calculated using data acquired by RT-NMR (adapted from Ref. [16••]). **(b)** Methionine oxidation of α-Synuclein was probed by RT-NMR in the presence of vesicles containing a fraction of peroxidized lipids; this provided quantitative data on oxidation of N-terminal Met residues (adapted from Ref. [24•]).

Figure 3



RT-NMR studies of GTPase oncoproteins. **(a)** RAS signaling revolves around the GTPase cycle, whereby stimuli activate RAS via exchange of GDP for GTP (a process facilitated by GEFs). Intrinsically slow GTP hydrolysis is enhanced by the activity of GAPs. Downstream signaling from RAS-GTP proceeds via numerous cellular effector proteins. **(b)** Overlay of GDP (black, PDB:4Q21) and GMMPNP (red, PDB:5P21) bound RAS structures. Conformational differences are particularly evident in the Switch regions (arrows), but are present throughout. **(c)** NMR-based analysis of nucleotide-dependent changes in RAS conformation. Overlay of 2D ^{1}H–^{15}N HSQC spectra reporting select backbone amide (top) resonances of RAS bound to GDP (black) or GMPPNP (red). **(d)** Cells expressing exogenous, full length p120GAP show increased hydrolysis activity (red) over control cells (black) or intrinsic (gray). Flag-tagged p120GAP was expressed using a Tet-inducible system, and confirmed by anti-Flag Western blotting (inset) (adapted from Ref. [30••]).

domains (Figure 3b). These conformational changes translate into easily discernable differences in NMR spectra, which enable facile discriminations between GTP-bound, and GDP-bound forms of the respective enzymes. GTP hydrolysis, in turn, transforms one spectral state into the other, which coincides with the time-dependent evolution of spectral features characteristic of the GDP-bound form (Figure 3c). By quantifying these changes in a time-resolved manner, individual GTP hydrolysis rates can be obtained. Marshall *et al.* used this approach to analyze GTPase kinetics of the RAS-homologue RHEB, by quantifying the transition of GTP-specific resonances to their GDP-specific counterparts [26]. Analogous RT-NMR approaches were employed to measure RHO [27] and ARF [28] GTPase activities, while a related methodology used ^{31}P-MAS RT-NMR to monitor ATP hydrolysis by a bacterial ABC transporter [29]. Recent applications of RT-NMR to study RAS itself have demonstrated how slow rates of hydrolysis keep RAS oncogenic mutants in the 'on' state [30••].

GTPases have typically weak intrinsic GTP hydrolysis activity, and require cellular GTPase-activating proteins (GAPs) for optimal function under physiological conditions. RT-NMR experiments with isotope-labeled GTPases and non-isotope-labeled GAPs can thus be employed to measure effects of GAPs on accelerating hydrolysis. By the same token, GDP-bound GTPases rely on interactions with cellular guanine nucleotide exchange factors (GEFs) to regain their GTP-bound states, effectively the opposite of GTP hydrolysis with regard to their spectral NMR features. Direct kinetic measurements of GTP hydrolysis by ^{15}N-RHEB in the presence of a purified, unlabeled GAP (TSC2) enabled the characterization of tuberous sclerosis-associated mutations in this co-factor [26]. Similar experiments with RAS measured GAP activity of a purified fragment of p120GAP, and GEF activity of a fragment of SOS1 [30••]. The influence of effector RAS-binding domains in reconstituted mixtures with GEFs and GAPs has also been studied by RT-NMR [31].

Building upon the idea that GTPase cycling can be observed in complex mixtures of associated network proteins, the activities of full-length GEFs and GAPs were also examined in whole cell extracts (Figure 3d). Purification of full-length SOS1 GEF remains a challenge; however, Smith *et al.* expressed wild-type SOS1 and a variety of disease-associated SOS1 mutants in mammalian cells, and directly studied their exchange activities in the resulting cell extracts [30••]. Further, activities from several overexpressed SOS1 variants were quantified directly in embryonic stem cell lysates [32]. It has also been possible to measure GAP activity from malignant peripheral nerve sheath tumor (MPNST) [30••] and colorectal cancer-derived cell extracts [33], and to dissect complex regulatory mechanisms of a microtubule-associated GEF for RHO GTPases [34,35]. Thus, RT-NMR has been successfully used to quantitatively report on activities and regulatory mechanisms of full-length enzymes, and to accurately assess the impact of disease-associated mutations.

One limitation of studying GTPase activities in cell extracts is the absence of intact membranes, which normally serve as key functional scaffolds for GTPase signaling processes. This is being addressed by tethering GTPases to nanodiscs; small discoidal lipoprotein complexes comprising a bilayer of 120–160 lipid molecules bound by apolipoproteins [36]. Mazhab-Jafari *et al.* anchored RHEB to such nanodiscs and demonstrated that membrane association markedly affected nucleotide exchange [37•]. Such types of native-like experimental systems offer great promise for future RT-NMR studies of membrane proteins.

Proline isomerization

Cis/*trans* isomerization of proline residues acts as a 'switch' mechanism to regulate ligand binding, autoinhibition, dephosphorylation, oligomerization and macromolecular assembly. Proline isomerization is invisible to most biochemical methods, however, NMR provides a powerful probe for this structural interconversion, and RT-NMR recently elucidated a role for proline isomerization in gene-3-protein (G3P) in filamentous phage infection [38]. The application of RT-NMR to monitor proline isomerization in protein folding studies was recently reviewed by Kumar and Balbach [39].

Real-time NMR monitoring of protein folding and assembly

RT-NMR has been used in several studies of protein folding and assembly processes, including the aforementioned work on proline isomerization [39,40]. Here, we highlight RT-NMR approaches that exploit the selective incorporation of ^{19}F fluorine to monitor protein folding and aggregation events [41], as well as ubiquitin chain assembly and disassembly, both in reconstituted ubiquitination systems and cell extracts [42]. The latter study provided insights into the activities of cellular ubiquitin ligases and deubiquitinases by delineating their site specificities at different substrate lysine residues. Site-selective ^{19}F labeling of proteins provides unique advantages for RT-NMR because it reduces the spectral complexity of observable NMR signals to one, or a few resonances. Given the high sensitivity of the ^{19}F nucleus (~80% that of protons), its virtual absence from physiological materials such as cell extracts, and its large chemical shift range, the use of incorporated ^{19}F as a site-specific spectroscopic probe for fast one-dimensional RT-NMR studies is highly attractive. In one such application, ^{19}F RT-NMR was employed to characterize the folding pathway of an intestinal fatty acid binding protein [43]. Li *et al.* were able to achieve a 'time resolution' of less than 5 seconds using an experimental strategy that combined stopped-flow techniques and NMR spectroscopy. This allowed identification of intermediate species along the folding pathway and measurement of their inter-conversion rates. ^{19}F RT-NMR also showed that aggregation of the islet amyloid peptide (IAPP) occurs without significant accumulations of pre-fibrillar species, in agreement with CD and fluorescence data [44], whereas the amyloid beta peptide (Aβ) populates at least six distinct oligomeric states along its route to aggregation [45]. Other RT-NMR applications addressed the oligomerization kinetics of the murine prion protein (PrP) and revealed that residues in the vicinity of two disulfide-forming cysteines are important for the initial encounter steps *en route* to aggregation, whereas the remainder of PrP only rigidifies when higher-order structures are formed [46]. Similarly, RT-NMR measurements were used to delineate site-specific effects of copper-induced aggregation of human α-synuclein [47].

Perspectives

Advances in high-field superconducting magnets, cryogenic probes, and pulse-sequence development have

greatly improved the sensitivity of biomolecular NMR spectroscopy over the past decade, and provided means to recording consecutive NMR experiments at ever shorter time intervals. Real-time NMR detection benefits greatly from rapid acquisitions of NMR spectra, which is further augmented by advancements in non-uniform sampling (NUS) and 'fast pulsing' experiments such as SOFAST-HMQC or BEST-HSQC [40]. Moreover, improvements in site-specific and amino-acid type-specific isotope labeling have helped to reduce spectral overlap and enabled RT-NMR studies of increasingly large and complex biological systems. New methods to assemble enzymatic scaffolding environments, including the use of different membrane mimetics and spatially oriented tethering complexes, allow us to better reconstitute defined *in vitro* reaction setups with 'native-like' properties, enabling greater environmental stability and, in turn, better experimental reproducibility. In parallel, several tailored protocols for the production of biologically active physiological environments such as enzyme-competent and cofactor-competent cell extracts suitable for RT-NMR applications have been developed [13••,15,30••,34]. Finally, several frontier in-cell NMR studies have been recently reported, aiming to obtain an even more detailed structural and functional understanding of proteins in live cells [48–50]. Future in-cell NMR approaches will include RT-NMR observations of enzymatic reactions, post-translational modifications, and protein folding events, with the goal to simultaneously monitor multiple biological activities in the context of intact cells, and in a virtually continuous fashion.

Conflict of interest statement

The authors declare no conflict of interest.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Purvis JE, Lahav G: **Encoding and decoding cellular information through signaling dynamics**. *Cell* 2013, **152**:945-956.

2. Eicher JJ, Snoep JL, Rohwer JM: **Determining enzyme kinetics for systems biology with nuclear magnetic resonance spectroscopy**. *Metabolites* 2012, **2**:818-843.

3. • Hall H, Cuellar-Baena S, Denisov V, Kirik D: **Development of NMR spectroscopic methods for dynamic detection of acetylcholine synthesis by choline acetyltransferase in hippocampal tissue**. *J Neurochem* 2013, **124**:336-346.

The authors demonstrate the possibility of monitoring ACh synthesis in rat brain homogenates by RT-NMR towards development of a non-invasive technique to assess the state of the cholinergic system in the brain. For detection, they used ^{15}N-choline and a pulse sequence designed to filter proton polarization by nitrogen coupling before ^{1}H NMR detection.

4. Lerche MH, Jensen PR, Karlsson M, Meier S: **NMR insights into the inner workings of living cells**. *Anal Chem* 2015, **87**:119-132 http://dx.doi.org/10.1021/ac501467x.

5. Harris T, Degani H, Frydman L: **Hyperpolarized ^{13}C NMR studies of glucose metabolism in living breast cancer cell cultures**. *NMR Biomed* 2013, **26**:1831-1843.

6. Chaumeil MM, Larson PE, Yoshihara HA, Danforth OM, Vigneron DB, Nelson SJ, Pieper RO, Phillips JJ, Ronen SM: **Non-invasive in vivo assessment of IDH1 mutational status in glioma**. *Nat Commun* 2013, **4**:2429.

7. Venkatesh HS, Chaumeil MM, Ward CS, Haas-Kogan DA, James CD, Ronen SM: **Reduced phosphocholine and hyperpolarized lactate provide magnetic resonance biomarkers of PI3K/Akt/mTOR inhibition in glioblastoma**. *Neuro Oncol* 2012, **14**:315-325.

8. Lodi A, Woods SM, Ronen SM: **Treatment with the MEK inhibitor U0126 induces decreased hyperpolarized pyruvate to lactate conversion in breast, but not prostate, cancer cells**. *NMR Biomed* 2013, **26**:299-306.

9. Wagstaff JL, Masterton RJ, Povey JF, Smales CM, Howard MJ: **^{1}H NMR spectroscopy profiling of metabolic reprogramming of Chinese hamster ovary cells upon a temperature shift during culture**. *PLoS ONE* 2013, **8**:e77195 http://dx.doi.org/10.1371/journal.pone.0077195.

10. Xue J, Isern NG, Ewing RJ, Liyu AV, Sears JA, Knapp H, Iversen J, Sisk DR, Ahring BK, Majors PD: **New generation NMR bioreactor coupled with high-resolution NMR spectroscopy leads to novel discoveries in *Moorella thermoacetica* metabolic profiles**. *Appl Microbiol Biotechnol* 2014, **98**:8367-8375.

11. Jensen PR, Karlsson M, Lerche MH, Meier S: **Real-time DNP NMR observations of acetic acid uptake, intracellular acidification, and of consequences for glycolysis and alcoholic fermentation in yeast**. *Chemistry* 2013, **19**:13288-13293.

12. Theillet FX, Smet-Nocca C, Liokatis S, Thongwichian R, Kosten J, Yoon MK, Kriwacki RW, Landrieu I, Lippens G, Selenko P: **Cell signaling, post-translational protein modifications and NMR spectroscopy**. *J Biomol NMR* 2012, **54**:217-236.

13. •• Theillet F-X, Rose HM, Liokatis S, Binolfi A, Thongwichian R, Stuiver M, Selenko P: **Site-specific NMR mapping and time-resolved monitoring of serine and threonine phosphorylation in reconstituted kinase reactions and mammalian cell extracts**. *Nat Protoc* 2013, **8**:1416-1432.

This paper outlines protocols for site-specific mapping and time-resolved monitoring of protein phosphorylation reactions using purified kinases and mammalian cell extracts. Included are guidelines for sample preparation and quantitative data collection using consecutive ^{1}H–^{15}N band-selective optimized-flip-angle short-transient (SOFAST)-heteronuclear multiple-quantum (HMQC) NMR experiments.

14. Liokatis S, Stützer A, Elsässer SJ, Theillet F-X, Klingberg R, van Rossum B, Schwarzer D, Allis CD, Fischle W, Selenko P: **Phosphorylation of histone H3 Ser10 establishes a hierarchy for subsequent intramolecular modification events**. *Nat Struct Mol Biol* 2012, **19**:819-823.

15. Rose HM, Stuiver M, Thongwichian R, Theillet F-X, Feller SM, Selenko P: **Quantitative NMR analysis of Erk activity and inhibition by U0126 in a panel of patient-derived colorectal cancer cell lines**. *BBA Protein Proteom* 2013, **1834**:1396-1401.

16. •• Cordier F, Chaffotte A, Terrien E, Préhaud C, Theillet O-X, Delepierre M, Lafon M, Buc H, Wolf N: **Ordered phosphorylation events in two independent cascades of the PTEN C-tail revealed by NMR**. *J Am Chem Soc* 2012, **134**:20533-20543.

The authors use RT-NMR to report on the order and kinetics of clustered multisite phosphorylation events. They identify seven sites modified by CK2 and GSK3β kinases, and two new sites on the PTEN C-terminal tail. They determine the sequence of phosphorylation reactions and calculate their apparent rate constants, and also assess their formation by endogenous kinases directly in extracts of human neuroblastoma cells.

17. Kosten J, Binolfi A, Stuiver M, Verzini S, Theillet FX, Bekei B, van Rossum M, Selenko P: **Efficient modification of alpha-synuclein serine 129 by protein kinase CK1 requires phosphorylation of tyrosine 125 as a priming event**. *ACS Chem Neurosci* 2014, **5**:1203-1208 http://dx.doi.org/10.1021/cn5002254.

18. Borcherds W, Theillet FX, Katzer A, Finzel A, Mishall KM, Powell AT, Wu H, Manieri W, Dieterich C, Selenko P *et al.*: **Disorder and residual helicity alter p53-Mdm2 binding affinity and signaling in cells**. *Nat Chem Biol* 2014, **10**:1000-1002.

19. Amata I, Maffei M, Igea A, Gay M, Vilaseca M, Nebreda AR, Pons M: **Multi-phosphorylation of the intrinsically disordered unique domain of c-Src studied by in-cell and real-time NMR spectroscopy**. *ChemBioChem* 2013, **14**:1820-1827.

20. Selenko P, Frueh DP, Elsaesser SJ, Haas W, Gygi SP, Wagner G: **In situ observation of protein phosphorylation by high-resolution NMR spectroscopy**. *Nat Struct Mol Biol* 2008, **15**:321-329.

21. Mayzel M, Rosenlöw J, Isaksson L, Orekhov VY: **Time-resolved multidimensional NMR with non-uniform sampling**. *J Biomol NMR* 2014, **58**:129-139.

22. Liokatis S, Dose A, Schwarzer D, Selenko P: **Simultaneous detection of protein phosphorylation and acetylation by high-resolution NMR spectroscopy**. *J Am Chem Soc* 2010, **132**:14704-14705.

23. Theillet F-X, Liokatis S, Jost JO, Bekei B, Rose HM, Binolfi A, Schwarzer D, Selenko P: **Site-specific mapping and time-resolved monitoring of lysine methylation by high-resolution NMR spectroscopy**. *J Am Chem Soc* 2012, **134**:7616-7619.

24. • Maltsev AS, Chen J, Levine RL, Bax A: **Site-specific interaction between α-synuclein and membranes probed by NMR-observed methionine oxidation rates**. *J Am Chem Soc* 2013, **135**:2943-2946.

The authors apply RT-NMR to observe Met oxidation in α-Synuclein in the presence of lipid vesicles containing peroxidized lipids. They demonstrate that N-terminal Met residues rapidly oxidize, whereas C-terminal Met residues remain unaffected. The results show that oxidation of Met1 reduces the rate of oxidation of Met5 and vice versa as a result of decreased membrane affinity of the partially oxidized protein.

25. Miotto MC, Rodriguez EE, Valiente-gabioud AA, Torres-monserrat V, Quintanar L, Zweckstetter M, Griesinger C, Ferna CO: **Site-specific copper-catalyzed oxidation of α-synuclein: tightening the link between metal binding and protein oxidative damage in Parkinson's disease**. *Inorg Chem* 2014, **53**:4350-4358.

26. Marshall CB, Ho J, Buerger C, Plevin MJ, Li G-Y, Li Z, Ikura M, Stambolic V: **Characterization of the intrinsic and TSC2-GAP-regulated GTPase activity of Rheb by real-time NMR**. *Sci Signal* 2009, **2**:ra3 http://dx.doi.org/10.1126/scisignal.2000029.

27. Gasmi-Seabrook GMC, Marshall CB, Cheung M, Kim B, Wang F, Jang YJ, Mak TW, Stambolic V, Ikura M: **Real-time NMR study of guanine nucleotide exchange and activation of RhoA by PDZ-RhoGEF**. *J Biol Chem* 2010, **285**:5137-5145.

28. Buosi V, Placial J-P, Leroy J-L, Cherfils J, Guittet É, van Heijenoort C: **Insight into the role of dynamics in the conformational switch of the small GTP-binding protein Arf1**. *J Biol Chem* 2010, **285**:37987-37994.

29. Hellmich Ua, Haase W, Velamakanni S, van Veen HW, Glaubitz C: **Caught in the act: ATP hydrolysis of an ABC-multidrug transporter followed by real-time magic angle spinning NMR**. *FEBS Lett* 2008, **582**:3557-3562.

30. •• Smith MJ, Neel BG, Ikura M: **NMR-based functional profiling of RASopathies and oncogenic RAS mutations**. *Proc Natl Acad Sci USA* 2013:110.

RT-NMR was used to obtain detailed mechanistic data on RAS cycling defects conferred by oncogenic mutations, or full-length RASopathy-derived regulatory proteins. The authors monitored the conformation of wild-type and oncogenic RAS variants in real time, showing distinct biochemical properties, and used whole cell lysates to monitor activity from full length GAPs and GEFs.

31. Smith MJ, Ikura M: **Integrated RAS signaling defined by parallel NMR detection of effectors and regulators**. *Nat Chem Biol* 2014, **10**:223-230.

32. Findlay GM, Smith MJ, Lanner F, Hsiung MS, Gish GD, Petsalaki E, Cockburn K, Kaneko T, Huang H, Bagshaw RD *et al.*: **Interaction domains of Sos1/Grb2 are finely tuned for cooperative control of embryonic stem cell fate**. *Cell* 2013, **152**:1008-1020.

33. Organ SL, Hai J, Radulovich N, Marshall CB, Leung L, Sasazuki T, Shirasawa S, Zhu C-Q, Navab R, Ikura M *et al.*: **p120RasGAP is a mediator of Rho pathway activation and tumorigenicity in the DLD1 colorectal cancer cell line**. *PLoS ONE* 2014, **9**:e86103 http://dx.doi.org/10.1371/journal.pone.0086103.

34. Meiri D, Marshall CB, Greeve Ma, Kim B, Balan M, Suarez F, Bakal C, Wu C, Larose J, Fine N *et al.*: **Mechanistic insight into the microtubule and actin cytoskeleton coupling through dynein-dependent RhoGEF inhibition**. *Mol Cell* 2012, **45**:642-655.

35. Meiri D, Marshall CB, Mokady D, LaRose J, Mullin M, Gingras A-C, Ikura M, Rottapel R: **Mechanistic insight into GPCR-mediated activation of the microtubule-associated RhoA exchange factor GEF-H1**. *Nat Commun* 2014, **5**:4857 http://dx.doi.org/10.1038/ncomms5857.

36. Denisov IG, Grinkova YV, Lazarides AA, Sligar SG: **Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size**. *J Am Chem Soc* 2004, **126**:3477-3487.

37. • Mazhab-Jafari MT, Marshall CB, Stathopulos PB, Kobashigawa Y, Stambolic V, Kay LE, Inagaki F, Ikura M: **Membrane-dependent modulation of the mTOR activator Rheb: NMR observations of a GTPase tethered to a lipid-bilayer nanodisc**. *J Am Chem Soc* 2013, **135**:3367-3370.

The authors take RT-NMR to a lipid bilayer to characterize RHEB nucleotide exchange and GTP hydrolysis. This revealed that GTPase domains interact transiently with the surface of the bilayer in preferred orientations, determined by the bound nucleotide.

38. Hoffmann-Thoms S, Weininger U, Eckert B, Jakob RP, Koch JR, Balbach J, Schmid FX: **Initiation of phage infection by partial unfolding and prolyl isomerization**. *J Biol Chem* 2013, **288**:12979-12991.

39. Kumar A, Balbach J: **Real-time protein NMR spectroscopy and investigation of assisted protein folding**. *Biochim Biophys Acta* 2014, **9**:1242-1250.

40. Rennella E, Brutscher B: **Fast real-time NMR methods for characterizing short-lived molecular states**. *ChemPhysChem* 2013, **14**:3059-3070.

41. Marsh ENG, Suzuki Y: **Using F-19 NMR to probe biological interactions of proteins and peptides**. *ACS Chem Biol* 2014, **9**:1242-1250.

42. Shekhawat SS, Pham GH, Prabakaran J, Strieter ER: **Simultaneous detection of distinct ubiquitin chain topologies by 19F NMR**. *ACS Chem Biol* 2014, **9**:2229-2236.

43. Li HL, Frieden C: **Observation of sequential steps in the folding of intestinal fatty acid binding protein using a slow folding mutant and F-19 NMR**. *Proc Natl Acad Sci USA* 2007, **104**:11993-11998.

44. Suzuki Y, Brender JR, Hartman K, Ramamoorthy A, Marsh ENG: **Alternative pathways of human islet amyloid polypeptide aggregation distinguished by F-19 nuclear magnetic resonance-detected kinetics of monomer consumption**. *Biochemistry* 2012, **51**:8154-8162.

45. Suzuki Y, Brender JR, Soper MT, Krishnamoorthy J, Zhou YL, Ruotolo BT, Kotov NA, Ramamoorthy A, Marsh ENG: **Resolution of oligomeric species during the aggregation of A beta(1-40) using F-19 NMR**. *Biochemistry* 2013, **52**:1903-1912.

46. Schlepckow K, Schwalbe H: **Molecular mechanism of prion protein oligomerization at atomic resolution**. *Angew Chem (International ed. in English)* 2013, **52**:10002-10005.

47. Binolfi A, Rodriguez EE, Valensin D, D'Amelio N, Ippoliti E, Obal G, Duran R, Magistrato A, Pritsch O, Zweckstetter M *et al.*: **Bioinorganic chemistry of Parkinson's disease: structural**

determinants for the copper-mediated amyloid formation of alpha-synuclein. *Inorg Chem* 2010, **49**:10668-10679.

48. Inomata K, Ohno A, Tochio H, Isogai S, Tenno T, Nakase I, Takeuchi T, Futaki S, Ito Y, Hiroaki H *et al.*: **High-resolution multi-dimensional NMR spectroscopy of proteins in human cells**. *Nature* 2009, **458**:106-109.

49. Hamatsu J, O'Donovan D, Tanaka T, Shirai T, Hourai Y, Mikawa T, Ikeya T, Mishima M, Boucher W, Smith BO *et al.*: **High-resolution heteronuclear multidimensional NMR of proteins in living insect cells using a baculovirus protein expression system**. *J Am Chem Soc* 2013, **135**:1688-1691.

50. Banci L, Barbieri L, Bertini I, Luchinat E, Secci E, Zhao Y, Aricescu aR: **Atomic-resolution monitoring of protein maturation in live human cells by NMR**. *Nat Chem Biol* 2013, **9**:297-299.