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Perspectives in Enzymology of Membrane Proteins by Solid-State NMR

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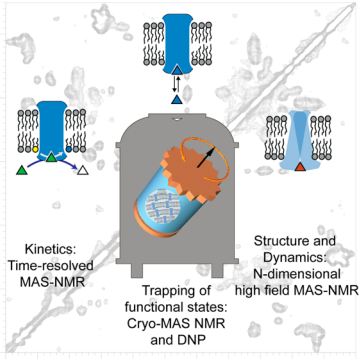
CONSPECTUS

Membrane proteins catalyze reactions at the cell membrane and facilitate the transport of molecules or signals across the membrane. Recently researchers have made great progress in understanding the structural biology of membrane proteins, mainly based on X-ray crystallography. In addition, the application of complementary spectroscopic techniques has allowed researchers to develop a functional understanding of these proteins. Solid-state NMR has become an indispensable tool for the structure–function analysis of insoluble proteins and protein complexes. It offers the possibility of investigating membrane proteins directly in their environment, which provides essential information about the intrinsic coupling of protein structure and functional dynamics within the lipid bilayer.



However, to date, researchers have hardly explored the enzymology of membrane proteins. In this Account, we review the perspectives for investigating membrane-bound enzymes by solid-state NMR. Understanding enzyme mechanisms requires access to kinetic parameters, structural analysis of the catalytic center, knowledge of the 3D structure and methods to follow the structural dynamics of the enzyme during the catalytic cycle. In principle, solid-state NMR can address all of these issues. Researchers can characterize the enzyme kinetics by observing substrate turnover within the membrane or at the membrane interphase in a time-resolved fashion as shown for diacylglycerol kinase. Solid-state NMR has also provided a mechanistic understanding of soluble enzymes including triosephosphate isomerase (TIM) and different metal-binding proteins, which demonstrates a promising perspective also for membrane proteins.

The increasing availability of high magnetic fields and the development of new experimental schemes and computational protocols have made it easier to determine 3D structure using solid-state NMR. Dynamic nuclear polarization, a key technique to boost sensitivity of solid-state NMR at low temperatures, can help with the analysis of thermally trapped catalytic intermediates, while methods to improve signal-to-noise per time unit enable the real-time measurement of kinetics of conformational changes during the catalytic cycle.

Introduction: Solid-State NMR on Enzymes

Understanding enzyme mechanisms requires the identification of intermediates, resolving their structure, and linking the kinetics of the catalyzed reaction to protein dynamics. To obtain such a "molecular movie" along the reaction coordinate is a highly sought-after goal, which could be obtained either by time-resolved X-ray crystallography[1] or solid-state NMR. The latter was suggested almost 2 decades ago,[2] and the general potential of solid-state NMR to probe substrate–enzyme complexes under equilibrium conditions was highlighted recently.[3] There has been significant progress in solid-state NMR. Very recent developments, as outlined below, will enable researchers to use this method to its full extent for resolving mechanistic details of (membrane) proteins, which are difficult to study otherwise.

The use of solid-state NMR for a mechanistic understanding of globular enzymes has been demonstrated extensively for triosephosphate isomerase (TIM), an efficient catalyst for the interconversion of ketone to an aldehyde involving various proton transfer steps. It shows enzymatic activity in microcrystalline form, which has enabled dynamic studies linking the rate of loop motions to its functional mechanism[4] and analyzing reactive intermediate states directly under steady-state conditions.[5] Ligand

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binding induced chemical shift changes have been used to identify residues involved in substrate binding.[6]

Solid-state NMR has also been used for metal binding enzymes. With application of ^{51}V MAS NMR, the CSA and quadrupole tensor of the vanadate cofactor in vanadium chloroperoxidase have been obtained from spinning side band analysis. Insight into coordination of the vanadate cofactor in the binding pocket[7] became accessible through modeling of the NMR data by density functional theory. A combination of REDOR and quantum chemical calculations also enabled determination of the substrate position in a peptide model of the nickel superoxide dismutase.[8] ^{13}C MAS NMR studies on precipitates of selectively labeled cytochrome P450 BM-3, a cytochrome P450 variant containing its own reductase domain and without a membrane anchor, revealed conformational changes within the substrate-binding pocket.[9,10] A promising perspective for the investigation of full length cytochrome P450 and cytochrome b5 has been given by incorporating them into lipid bilayers for MAS and oriented sample solid-state NMR[11] or by preparing precipitates.[12]

Triggered by solid-state NMR resonance assignment and structure determination of microcrystalline proteins,[13] good progress has also been made for integral membrane proteins and membrane bound enzymes. DsbB, a membrane bound enzyme consisting of four transmembrane helices catalyzes the transfer of two electrons from two cysteines to the redox-active cofactor ubiquinone. Solid-state NMR provided evidence for a charge transfer from DsbB to ubiquinone within the membrane bound complex based on chemical shift changes.[14] Extensive assignment of even larger 7TM receptors has been reported for different bacterial retinal proteins such as sensory rhodopsin and proteorhodopsin.[15−17] The possibility for *de novo* structure determination was shown for the trimeric autotransporter adhesin YadA in microcrystalline preparations,[18] while a backbone structure of the human chemokine receptor CXCR1 in phospholipid bilayers has been reported.[19] Especially the latter case is an example for the number of hurdles that one has to overcome for membrane protein studies, including overexpression of a human GPCR in *Escherichia coli*, functional refolding from inclusion bodies and reconstitution, chemical shift assignment and structure determination including long-range constraints via relative helix orientations.[20]

The availability of structural information and biochemical data enables enunciatiation of well-founded hypotheses on the functional mechanism of membrane proteins, which can be tested experimentally by solid-state NMR. The probably best-known example for this hypotheses driven research is

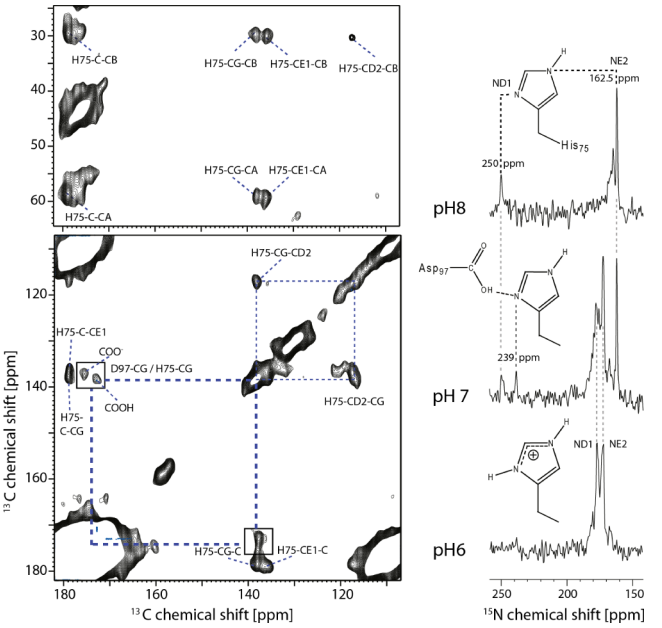

FIGURE 1. A pH-dependent His-Asp cluster involved in activity regulation and a characteristic feature of eubacterial light-driven proton pumps has been identified by solid-state NMR in green proteorhodopsin.[25] The close proximity of His75 and Asp97 was found by ^{13}C−^{13}C correlation spectroscopy, while the nature of the H-bond formation between both side chains was analyzed by ^{15}N-MAS NMR. Reproduced from ref 25. Copyright 2011 American Chemical Society.

the work on the proton transfer mechanism of bacteriorhodopsin, which is also the earliest example for solid-state NMR studies on membrane proteins (for a review, see ref 21). More recent studies, among many other applications, provided a better molecular understanding of ion channels.[22,23] For example, solid-state NMR data yielded insight into the possible mechanism of proton conduction in the M2 channel involving a H-bonded network formed by four histidines.[24] In another case, the pH-dependent formation of a His-Asp cluster close to the active site of a membrane protein and its role for the pH-dependent proton transfer has been found in proteorhodopsin, a light-driven proton pump (Figure 1).[25]

Real-Time Solid-State NMR To Investigate Enzyme Kinetics

In addition to a structural characterization, the kinetics of enzyme-catalyzed reaction has to be analyzed, which is usually done by observing substrate turnover in a time-resolved manner. In case of soluble substrates, a multitude of methods can be applied, but difficulties are encountered if these reactions involve, for example, lipids or other hydrophobic compounds associated with the membrane. This is, for example, true in the case of membrane bound kinases involved in lipid regulation. These proteins transfer the

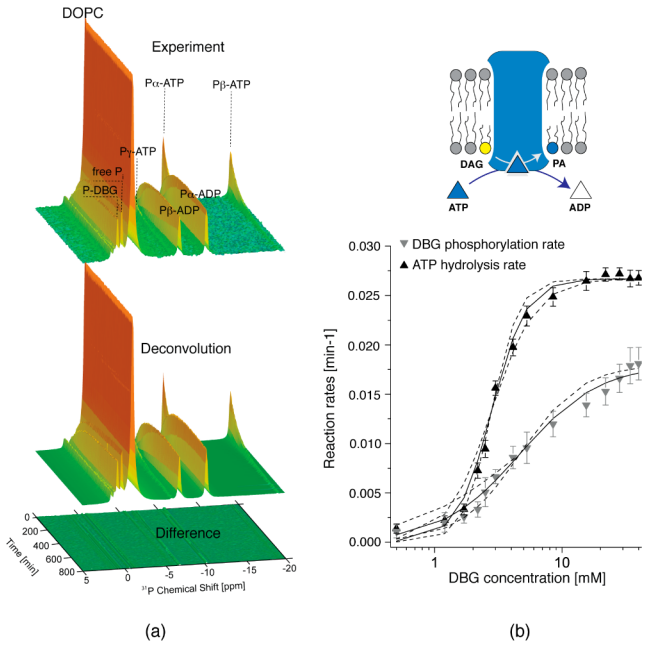

FIGURE 2. Time-resolved ^{31}P MAS NMR spectra reveal the full enzymatic activity of the integral membrane protein diacylglycerol kinase reconstituted into DOPC lipid bilayers[26] (a). A series of pseudo-2D data sets has been recorded for varying ATP and DBG concentrations and completely deconvoluted to obtain rate constants. These rates for DAGK catalyzed ATP hydrolysis and DBG are plotted as a function of DBG concentration and can be analyzed using kinetic models (b).

γ-phosphoryl group from ATP to a lipid acting as second messenger, e.g. diacylglycerol. The possibility to follow these reactions within lipid bilayers by time-resolved MAS NMR has been demonstrated for diacylglycerol kinase (DAGK) from *E. coli*.[26] This protein phosphorylates various variants of diacylglycerol to phosphatidic acid at the expense of ATP (for a review, see ref 27). While the turnover of ATP and P_i could be observed in the aqueous phase, the lipid product accumulates within the membrane. Both phases are simultaneously accessible by ^{31}P MAS NMR as shown in Figure 2a. Diacylglycerols with varying chain lengths have been used as lipid substrates. The determination of reaction rates from the observed time traces in Figure 2a as a function of ATP or lipid substrate concentration enables elucidation of kinetic parameters. Figure 2b shows the dependence of the ATP hydrolysis rate as well as the phosphorylation rate of 1,2-dibutyrylglycerol (DBG), a diacylglycerol variant, as a function of DBG concentration. The data reveal a surprisingly loose coupling between ATP consumption and lipid phosphorylation for this particular substrate, which is difficult to detect by other methods. Furthermore, a higher degree of cooperativity for ATP hydrolysis compared with substrate phosphorylation, at least for short-chain DBG, was obtained (Figure 2b). This study demonstrates that time-resolved solid-state NMR can monitor enzymatic reactions at the membrane interface in atomic detail. It was found that ATP hydrolysis is uncoupled from lipid phosphorylation by *ortho*-vanadate indicating that a specific phosphoryl transfer pathway might have been blocked. In a similar study, basal ATPase activity of an ABC transporter reconstituted into liposomes has been observed by time-resolved ^{31}P MAS NMR.[28]

Although conceptually straightforward, such kinetic experiments require careful design to minimize the dead time and maximize time resolution. The limiting factors are (A) the mechanism used for initiating the reaction and (B) the time resolution achievable by NMR. Furthermore, the observation of substrate turnover provides functional insight via kinetic analysis, while a time-resolved detection of the formation of enzyme–substrate complexes enables a direct mechanistic "movie" of the protein's activity.

A. Initiating a Reaction. The most straightforward approach for initiating an enzymatic reaction is an initial mixing step outside of the spectrometer directly within the MAS rotor followed by rapid closure and transfer of the rotor into the RF coil of the MAS probe head and spinning up the sample to the desired spin rate. This procedure will always result in a certain dead time. An elegant option has been developed in solution-state NMR, where mixing is performed directly in the sample tube within the RF coil.[29] Unfortunately, it is difficult to adapt this approach to MAS NMR due to the closed design of the MAS rotor. However, the use of photolabile caged compounds offers the convenient possibility to circumvent the initial mixing problem as demonstrated by a time-resolved ^{31}P MAS NMR study on the light-induced release of ATP from caged NPE–ATP in frozen solution.[30] Data were acquired under continuous illumination and revealed kinetic as well as mechanistic details of this intramolecular redox reaction. A more complex demonstration of this approach was presented by probing the enzymatic activity of DNA ligase, an enzyme that catalyzes the formation of a phosphodiester bond needed for facilitating the joining of DNA bonds.[31] The reaction was triggerd *in situ* by the photorelease of caged Mg^{2+}, which was homogenously mixed with ATP and the DNA ligase in a heparin matrix for stabilization. The subsequent ATP turnover has been monitored at 245 K. At this temperature, the reaction was slow enough to detect an intermediate tentatively assigned to a pentacoordinated phosphorane complex. These studies illustrate that time-resolved solid-state NMR also offers interesting opportunities for mechanistic studies on soluble proteins, especially for the detection of

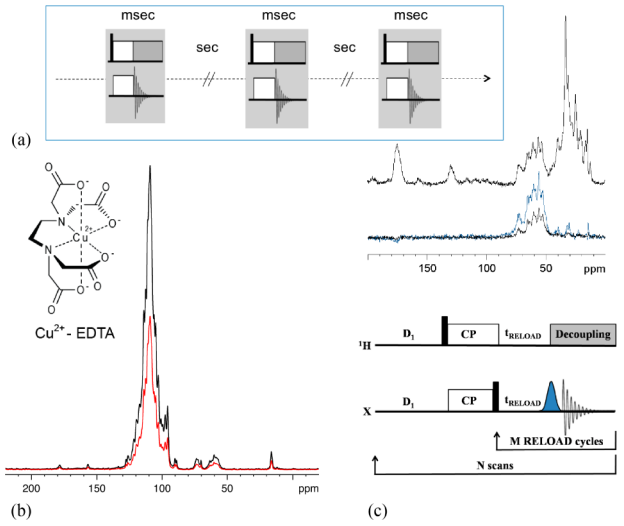

FIGURE 3. The total time required for NMR experiments is dominated to 90% by an interscan delay during which the spin system returns to equilibrium (a). The experimental time can be shortened by reducing ^{1}H-T_1 through sample doping, for example, with Cu^{2+}-EDTA,[34] as illustrated here for the 7TM membrane protein proteorhodopsin reconstituted in lipid bilayers (b). The sample has been doped with 15 mM Cu^{2+}-EDTA resulting in a 4-times shorter ^{1}H-T_1 and hence 2-fold better S/N within the same experimental time (unpublished data). Band selective excitation is another possibility to accumulate magnetization faster. An implementation for CP-MAS is given by the RELOAD experiment[42] (c). Here, ^{13}C magnetization is rapidly accumulated *M*-times by a selective 90° pulse after CP enhanced magnetization is stored along *z*. This way, a band selective 2−3 fold-signal enhancement per time unit can be achieved, as illustrated for the Cα region in proteorhodopsin.

intermediate states. In case of membrane proteins, it is especially important to ensure efficient mixing of the proteoliposomes with the particular substrates in order to facilitate binding site accessibility in the sample pellets applied for MAS NMR.

(B). Time Resolution in Real-Time Solid-State NMR Studies. Solid-state NMR is a relatively insensitive technique. It is therefore important to find conditions for real-time experiments under which sufficient signal-to-noise per time unit can be obtained. This is possible either by slowing the reaction or by accelerating the NMR experiment itself. Lowering the temperature is an obvious possibility to slow the reaction rate and might help to accumulate intermediates, but the use of inert cryosolvents such as glycerol is required to prevent sample freezing. Accelerating the NMR experiment itself can be achieved by shortening the relaxation delay needed to equilibrate Boltzmann magnetization (Figure 3a). The time required for these interscan delays determines essentially the time resolution of 1D real-time NMR experiments and is in the order of seconds accounting for 90% of the total measurement time. Therefore, time resolution could be improved by shortening the relaxation delay, for instance, by Ernst angle excitation, by paramagnetic relaxation enhancement, or by band selective excitation.

Ernst angle excitation[32] has been especially useful in real-time solution-state NMR. It is based on the fact that magnetization returns faster to equilibrium for smaller flip angles. Although easy to apply, the gain in better time resolution is paid for by a loss in magnetization, and it is therefore only applicable to cases that are not limited by signal-to-noise.

The experimental time can also be shortened significantly by doping the sample with paramagnetic relaxation agents, which reduce the nuclear spin−lattice relaxation time.[33] For biomolecular solid-state NMR applications, especially, the use of Cu^{2+}-EDTA has been shown to be highly effective to improve signal-to-noise per time unit for crystalline proteins[34] or membrane proteins.[35] This approach is illustrated in Figure 3b. It has also been shown that copper-chelating lipids can be used as relaxation agents, which could be of particular interest for membrane protein studies.[36] Shortening of T_1 has also been observed in metalloproteins such as superoxide dismutase (SOD), enabling fast detection of MAS NMR spectra on these samples.[37] One essential drawback of reducing the interscan delay in most solid-state NMR experiments is sample heating due to the necessity of high-power proton decoupling. However, heating can be reduced by special probe heads with reduced E-fields[38] or by the use of low-power decoupling in combination with sample perdeuteration[39] and very high MAS spin rates (>40 kHz).[37] Especially sample perdeuteration also enables direct proton detection supporting even faster data acquisition.[39]

A third strategy for improving time resolution relies on band selective excitation as shown for SOFAST-NMR[40] and COST-HSQC[41] in solution-state NMR. A selectively excited subpopulation of spins returns to equilibrium much faster than the bulk spin system due to large spin temperature differences between the small number of excited and the large pool of nonexcited spins. Unfortunately, this approach cannot be directly transferred to solid-state NMR since initial magnetization is usually created via proton excitation through cross-polarization and proton resonances are homogeneously broadened preventing selective excitation. A solution has been demonstrated by RELOAD-CP as shown in Figure 3b.[42] In this experiment, ^{13}C magnetization is enhanced by a cross-polarization step and stored along the *z*-axis. Then, a selective 90° pulse reads out fast and repetitively ^{13}C magnetization until it has decayed to the Boltzmann level after which the spin system is "reloaded"

by another cross-polarization step. In this way, a band selective 2−3-fold signal enhancement per time unit can be achieved.

For obtaining site-specific information, multidimensional experiments are needed. In addition to the before-mentioned approaches, faster data acquisition can be achieved, for example, by nonuniform sampling,[43] reduced data dimensionality,[44] simultaneous recording using multiple receivers,[45] or covariance data processing.[46] While the experimental conditions can be tuned in a way that the time step needed for each increment is negligibly small compared with the observed reaction, kinetic data can also be extracted from the indirect dimension through the analysis of peak shapes and amplitudes. This approach has been developed originally in solution-state NMR for folding studies,[47] but its usability has also been demonstrated by following slow protein aggregation by MAS NMR.[48]

Perspectives for Investigating Enzymes by Rapid Freeze-Quenching Solid-State NMR

Despite all methods to shorten the interscan delay, the time resolution of real-time solid-state NMR can be still limited by the available signal-to-noise. A potential solution to this problem is given by time-resolved freeze quenching of the reaction of interest in combination with solid-state NMR.[2,49] It can be envisaged that in the near future thermal quenching approaches will also benefit from recent progress in the field of dynamic nuclear polarization (DNP), which provides major sensitivity enhancement in the solid state at low temperatures (for a comprehensive review, see ref 50). For the DNP cross effect, polarization transfer from the electrons to the nuclear spins is most efficient when biradicals with strongly dipolar coupled paramagnetic centers such as TOTAPOL are used.[51] A basic experimental scheme is shown in Figure 4a: A high power cw microwave source, usually a gyrotron, is coupled to an NMR spectrometer. The microwave frequency matches the electron Larmor frequency in the NMR magnet (e.g., 260 GHz vs 400 MHz at 9 T). For MAS NMR, microwaves are guided directly to the rotating sample through a waveguide as illustrated in Figure 4b. Commercial low-temperature MAS probe heads work routinely at 100 K, but DNP supported MAS NMR studies at 70−90 K at fields of up to 700 MHz/460 GHz using high power gyrotrons as microwave source[52] or even at 25 K at 400 MHz/260 GHz with a low power microwave source[53] have been reported.

DNP enables polarization transfer from electrons to protons followed by classical cross-polarization to ^{13}C or ^{15}N. A number of biomolecular samples were studied including frozen solutions of soluble biomolecules, as well as membrane

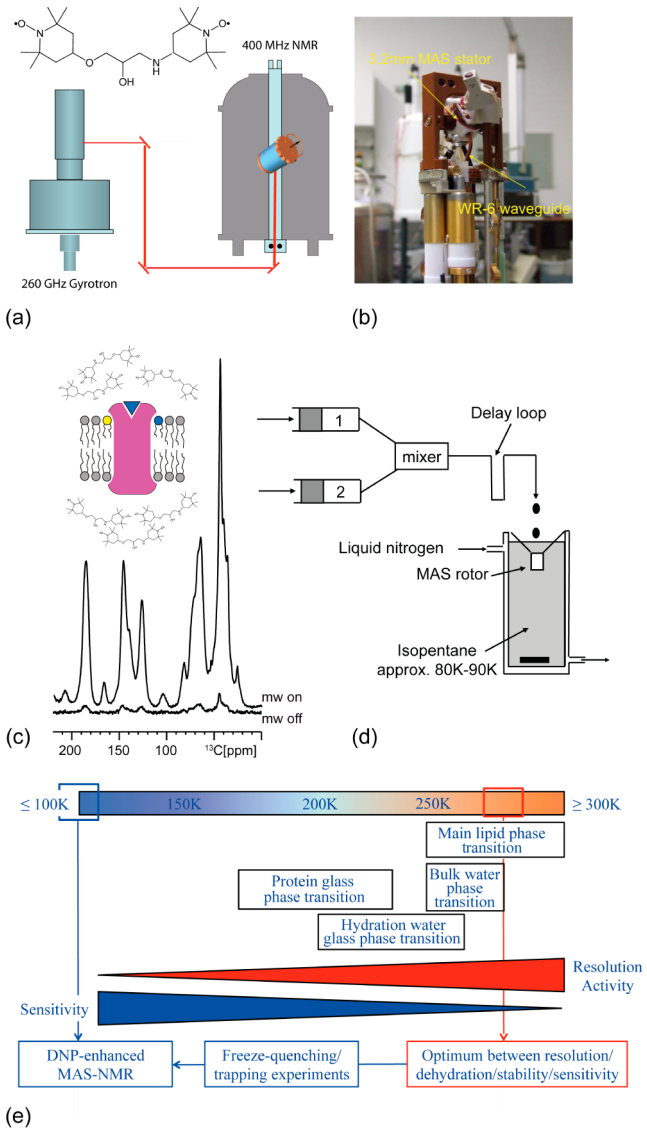

FIGURE 4. Schematic setup for DNP-enhanced MAS NMR (a). A high power cw microwave source (gyrotron) is coupled via a waveguide to the sample in the NMR spectrometer.[50] Samples have to be doped with polarizing agents such as the biradical TOTAPOL.[51] For MAS NMR, the microwave is directly guided through the coil at the rotating sample via a waveguide as shown here for a modified Bruker low temperature MAS probehead (b). DNP-enhanced ^{13}C MAS NMR on [^{13}C-His-Tyr]-DAGK reconstituted into DMPC lipid bilayers (20 mM TOTAPOL, 20% glycerol) shows a 20-fold signal enhancement at 100 K (c). Basic scheme for rapid freeze quenching of proteins for solid-state NMR[49] (d). Samples are rapidly mixed and quickly sprayed into the MAS rotor stored in a cryogen bath at low temperature. Delay loops can be used for "aging" of the quenching product. The sample undergoes a number of complex phase transitions during cooling/freezing to reach the temperature needed for DNP or thermal trapping (e).

proteins such as bacteriorhodopsin. For the latter, a signal enhancement of 40-fold at 90 K and 9 T was reported enabling 2D experiments on photo-intermediates, which would not be possible otherwise.[54] An example for a membrane bound

enzyme is shown in Figure 4c. Diacylglycerol kinase reconstituted in lipid bilayers has been suspended in a water/glycerol matrix with 20 mM TOTAPOL. A 20-fold signal enhancement is obtained at 100 K and 9 T. Other examples on membrane proteins with noncovalently bound isotope labeled ligands include the SEC translocon in complex with a signal peptide,[55] as well as acetylcholine receptor with bound neurotoxin II.[56]

As outlined above, the current mechanisms used for solid-state NMR signal enhancement by DNP work best at 100 K and below. This is also the temperature range required for thermal quenching of reactions, which would allow accumulating sufficient signal-to-noise for each interval. A sufficient time resolution can be achieved as long as the rate of freezing is much faster than the reaction of interest. The basic experimental layout[2] is shown in Figure 4d. Enzyme and substrate are rapidly mixed and quickly frozen by spraying them into a MAS rotor stored in secondary cryogen kept at temperatures between 85 and 140 K. For the first experiments, a dead time of approximately 6 ms was reported, which was very much reduced in recent years down to the range of 100 μs by optimized experimental designs.[49,57] This approach was successfully used for detecting transient enzyme-intermediate complexes of 5-enolpyruvylshikimate 3-phosphate synthase,[2,58] as well as protein folding intermediates.[49]

Although this approach appears very promising, some experimental aspects need to be considered for future applications. So far, all demonstrations except for bacteriorhodopsin were carried out on thermally quenched soluble proteins. In the case of membrane proteins, the use of liposomal suspensions might cause a challenge for rapid mixing due to high viscosity and a possible reduced binding site accessibility. If the latter situation poses a severe issue, the usage of nanodiscs might offer a potential solution.[59] Furthermore, the temperature dependence of buffers has to be taken into account.[60] Their pH can considerably change at low temperatures due to cooling induced precipitation of buffer components and enthalpic effects on the protonation equilibrium. However, if the rate of freezing used for thermal quenching is faster than the effects induced by thermal pH changes, their influence is probably less dramatic.

A multitude of phase transitions occur during cooling/freezing (Figure 4e). Proteins undergo a glass phase transition at around 200 K, which is associated with a significant change in molecular flexibility or "softness".[61] Below 250−300 K, most lipids switch from the liquid crystalline into the solid/gel phase, depending on chain length and degree of saturation. The freezing point of bulk water can be altered by buffer conditions but is found around 273 K, while water in the protein's hydration shell does not crystallize and turns into a glass phase approximately below 240 K. Therefore, the influence of protein, solvent, and lipid phase transitions occurring during the cooling/freezing process on protein integrity, NMR line width, and DNP enhancement has to be considered.

Cooling can cause partial changes in structure and activity of proteins due to altered properties of the aqueous solvent, changes in pH, H-bonds, and weaker hydrophobic interactions. These are usually fully reversible. In contrast, freezing results in dehydration due to ice formation, which could denature the protein, although these effects are also often reversible. In general, the use of cryoprotectants such as glycerol and fast freezing (>10^5 K/s) prevents hexagonal ice formation and allows preservation of the protein integrity during the freezing process. The effect of cooling/freezing on solid-state NMR line shapes has been investigated for frozen solution of soluble proteins,[62] protein microcrystals,[63] and membrane proteins.[64] When the temperature is lowered, the system goes more or less rapidly through the phase transitions shown in Figure 4e. In all cases, broadening of resonance lines has been observed at lower temperatures.

Best resolution for frozen solutions of proteins or nucleotides has been shown just below the freezing point of bulk water,[62,65] but line width increases at temperatures below and is dominated by inhomogeneous broadening. Similar observations have been reported in a number of cases for membrane proteins, where the optimum between resolution and CP efficiency is often achieved above the freezing point of water.[15,23,64,66] In both soluble and membrane proteins, hydration water has been found to be essential for protein dynamics and resolution at temperatures down to 240 K.[62,64] In the context of freeze quenching experiments, it seems favorable to rapidly pass these phase transitions and trap the samples below 150 K in a more homogeneous glass phase. Interestingly, solvent free samples, such as crystalline tripeptides, do not show a significant line broadening at low temperatures, and also membrane protein samples selectively labeled at positions inside their hydrophobic core provided promising resolution.[54,67]

Conclusions

Membrane bound enzymes offer an exciting but challenging area of research. For understanding their functional mechanism, kinetic and dynamic data and information on electronic states in the catalytic center are needed. Solid-state NMR tools have been developed to tackle these

questions, and membrane proteins become increasingly available at quantitative amounts. Most of the problems discussed here apply not only to enzymes but also to transporters and receptors, which, in some sense, "catalyze" transduction of matter or information across the membrane. Optimism seems justified in expecting important contributions from solid-sate NMR in resolving the functional mechanism of membrane proteins and especially membrane bound enzymes in the future.

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BIOGRAPHICAL INFORMATION

Sandra J. Ullrich studied Biochemistry at Universität Leipzig and Goethe Universität Frankfurt and received her Diploma in 2008. She has been working for her Ph.D. from 2008−2013 in the Glaubitz laboratory at the Goethe Universität. Her research interests are focused on membrane protein biophysics, lipid regulators, receptors, and solid-state NMR. She has been awarded with a Scholarship by the Studienstiftung des Deutschen Volkes.

Clemens Glaubitz, Professor of Biophysical Chemistry at Goethe Universität Frankfurt, received his Diploma in Physics from Universität Leipzig in 1994 and his Ph.D. in 1998 in Biochemistry from Oxford University. After conducting postdoctoral research at Oxford University and Stockholm University, he became junior research group leader at the FMP Berlin before joining Goethe Universität Frankfurt in 2002. His research is focused on mechanistic studies on membrane proteins, especially retinal proteins, transporters, receptors, and kinases. His main method is solid-state NMR combined by complementary biochemical and biophysical approaches. He has been a Rhodes scholar at Oxford University, received an Emmy Noether Research Grant and is director of the Frankfurt Centre for Biomolecular Magnetic Resonance.

FOOTNOTES

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The authors declare no competing financial interest.

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