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Protein deuteration *via* algal amino acids to circumvent proton back-exchange for ^{1}H-detected solid-state NMR†

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With perdeuteration, solid-state NMR spectroscopy of large proteins suffers from incomplete amide-proton back-exchange. Using a 72 kDa micro-crystalline protein, we show that deuteration exclusively *via* deuterated amino acids, well-established in solution to suppress sidechain protonation without proton back-exchange obstacles, provides spectral resolution comparable to perdeuterated preparations at intermediate spinning frequencies.

Protein perdeuteration has evolved as a fundamental technique in current NMR-based structural biology. Initially introduced for solution NMR, perdeuteration has significantly improved the relaxation properties of ^{1}H spins and heteronuclei. Fast magic-angle spinning (MAS) solid-state NMR has emerged as a potent alternative to NMR-based characterization of protein structure, dynamics, and interactions only more recently.[1,2] Compared to traditional ^{13}C-detected experiments, ^{1}H detection has become very popular due to its higher inherent sensitivity. Extensive deuteration of proteins with observation of only a few remaining protons, *e.g.* on amide-, methyl- or other side-chain sites, has proven to be a viable way to obtain high resolution and sensitivity.[3–6] Recently, important prospects have arisen from the favourable properties of perdeuterated, micro-crystalline samples in conjunction with increasingly complex pulse sequences.[7–10]

As much as perdeuteration has become indispensable for NMR, it has been imposing severe drawbacks for sample preparation, in particular high costs, low yields, and incompatibility with most potential expression systems. Equally importantly, for efficient amide proton back-exchange, high-molecular-weight proteins with a substantial hydrophobic core usually require unfolding using chemical denaturants followed by renaturation in H_2O. This is, however, not always feasible, as for many proteins unfolding/refolding results in a major decrease of catalytic activity and/or causes sample loss. Two approaches are usually used to partially evade this bottleneck. One is dispensing deuteration altogether and using ever higher magic-angle spinning frequencies to counteract the line broadening that results from the dense proton network. Despite the benefits of smaller rotor diameters required, however, these are associated with a certain impact on the sensitivity of the experiments compared to back-exchanged perdeuterated samples in 1.3 mm rotors. In addition, even at 100 kHz MAS, protonated samples tend to have slightly less favourable amide ^{1}H resonance linewidths, transfer efficiencies, and coherence lifetimes than deuterated samples at 50–60 kHz. For membrane proteins, ''inverted fractional deuteration'', iFD, has been used as an alternative to quantitatively incorporate amide protons while maintaining partial deuteration of the side chains.[11] This technique involves protonated water while exclusively deuterating the carbon source (usually glucose) of a minimal medium. For membrane proteins, which tend to show slightly broader linewidths compared to micro-crystalline proteins, compatibility of the latter even with larger rotors (desired to increase the number of spins in the rotor) and their associated moderate magic-angle-spinning rates has been demonstrated. However, due to the rather high remaining proton content (see below), in addition to the impact on coherence lifetimes and transfer efficiencies,[1,3] the added homogeneous proton line broadening may compromise the exquisite spectral advantages usually obtained using micro-crystalline samples.

Successful assessment of high-molecular-weight proteins at the frontline of the currently accessible protein complexity is bound to sharp resonances and high sensitivity, generally afforded by sufficient sample amounts of deuterated micro-crystals.[6,7] As a consequence, any additional line broadening due to added proton content poses a new challenge for the feasibility of conducting a comprehensive NMR characterization of increasingly complex research targets. The solution

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NMR field has invested ample efforts to harness high degrees of residual deuteration while still avoiding the conundrum of proton back exchange. One eminent, well-established procedure, compatible even with insect cells and expression in yeast, is based on perdeuterated amino acid mixes, which can be obtained in an affordable way from algal extracts.[12–15] Here we show that exploiting the existing approach of using H_2O with such perdeuterated, $^{15}N/^{13}C$-labeled algal extracts instead of glucose-based minimal media, while circumventing the requirement for proton back-exchange, can maintain the very narrow resonance lines associated with well-behaved perdeuterated micro-crystalline proteins under MAS. The approach is demonstrated to warrant assessment of non-water-exchangeable regions while maintaining favourable sensitivity and resolution for the 72 kDa enzyme tryptophane synthase as a representative high-molecular-weight micro-crystalline protein target with a poorly water-accessible hydrophobic core.

The need for proton back-exchange into previously deuterated amide sites in conventional (perdeuteration) approaches is derived from the complete avoidance of protons in D_2O-based buffers (Fig. 1A). In order to provide the highest deuteration level possible while still affording an expression system based on H_2O, we turned to fully perdeuterated amino acids, which can be obtained commercially in the form of algal amino acid mixes (not used here) or the whole soluble extracts from algae cultures (used here, Fig. 1B).[12] Such extracts are more often employed as *supplements* to boost expression in glucose-based minimal media (also compare a recent study on proton back-exchange by Napoli *et al.*[16]) but can also be used as the *sole* carbon source, *e.g.* in the case of SILEX® to generate labelling schemes facilitating H^α detection.[17] In order to compare the deuteration levels obtained for the algal extract as a function of sidechain carbon position in particular with the established iFD approach we expressed the SH3 domain of chicken α-spectrin as a well-characterized, low-molecular-weight model system in three different settings and pursued a series of solution NMR assessments. On one hand, we prepared a culture by exclusively mixing H_2O and commercial ISOGRO® powder according to Löhr *et al.*[12] In addition, we prepared an iFD sample according to Medeiros–Silva *et al.*,[11] and finally, a non-deuterated (u-$^{13}C/^{15}N$) sample was produced according to standard protocols (see all preparative details in the ESI†). The samples were assessed in terms of protonation patterns according to previously established principles.[5] In brief, the H^α protonation levels were quantified using a 2D H(N)(CO)CA experiment without proton decoupling in the C^α dimension, which allows to quantitatively compare the intensities of proton-coupled C^α doublets stemming from CH moieties with the intensities of the CD-derived singlet peaks. Additional ^1H-decoupled 2D H(N)(CO)CA spectra were recorded to transfer resonance assignments. The protonation of other sidechain carbons was determined using a simple ^{13}C constant-time HSQC with both, proton and deuterium decoupling upon indirect evolution, quantitatively comparing the two partially deuterated samples with the non-deuterated one. Fig. 1C and D compare the proton content at the H^α position, other aliphatic sites, as well the overall protonation of the residues as a function of the amino acid type for the two labelling schemes. Note that not all amino acid types are present or sufficiently dispersed in the SH3 domain, but the general trend is unambiguous.

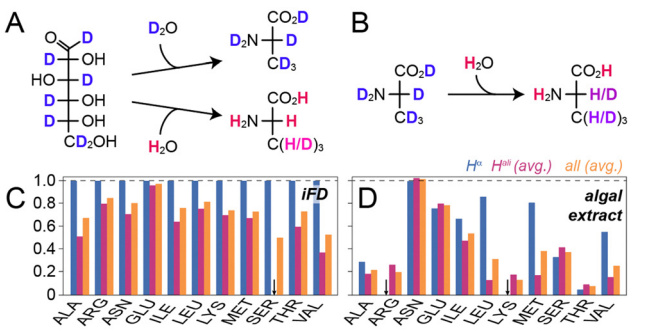

Fig. 1 Sidechain deuteration using deuterated algal extracts in comparison to sidechain deuteration from minimal media. (A) Partial conversion of water protons into fractionally deuterated amino acids in minimal media based on deuterated glucose (top: perdeuteration, bottom: iFD). (B) Upon employing deuterated amino acids, a high degree of eventual sample deuteration obtained. (C) Quantitative protonation chart for deuteration based on glucose- and H_2O-containing minimal medium (iFD labelling). (D) Protonation chart for water/algal extract-based sidechain deuteration. Amino acids/amino acid mixtures in A/B are generically represented by Ala. The degree of deuteration in (A) and (B) is tendentially visualized by a blue (deuterated) to red (protonated) colour gradient. Black arrows in (C) and (D) indicate missing peaks, *i.e.*, complete deuteration of those sites, resulting in overall lower protonation ratios of the sample.

In order to test the feasibility of $^1H^N$-detected experiments without proton back-exchange upon algal amino acid labelling, we prepared two micro-crystalline samples of *S. typhimurium* tryptophan synthase (TS), a large (2 × 72 kDa) αββα dimer of dimers with a substantial hydrophobic core, according to respective established protocols.[7,18] The first sample was produced in a perdeuterated and back-exchanged fashion, the other using H_2O and ISOGRO algal amino acids according to Löhr *et al.*[12] (see preparative and experimental details in the ESI†). The samples were assessed in 1.3 mm solid-state NMR rotors, spun at 55 kHz in a 700 MHz magnet at ~25 °C. (Beyond the sensitivity considerations regarding smaller rotors described in the introduction, larger rotors (1.9 mm) either incur broader proton lines and reduced transfer efficiencies or – in case of a stochastic introduction of amide deuterons, *e.g.*, to 30% – correlations involving more than one amide would be compromised, *i.e.* through-space correlations or sequential amide-to-amide correlations.[1]) At first, we wondered whether the back-exchange problem encountered for TS in previous work could in fact be alleviated by the algal amino acid labelling. We hence recorded proton-detected 3D triple-resonance experiments for the perdeuterated and proton-back-exchanged protein as well as for the algae-based sample. Fig. 2A shows representative slices from 3D hCONH experiments for the two samples. As we hoped for, the additional appearance of correlation peaks is evident. Fig. S2 and S3 (ESI†) provide more of such slices (including cross sections), in total

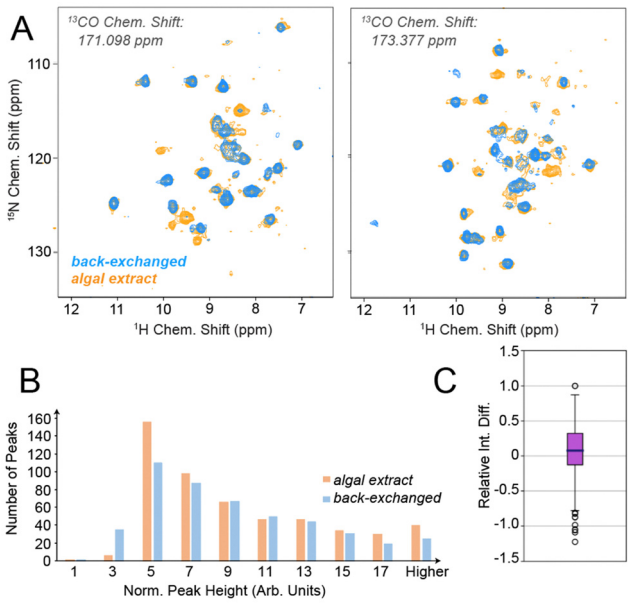

Fig. 2 Capturing of water-inaccessible residues in TS through algal amino acid labelling. (A) Representative slices of a 3D hCONH experiment recorded for TS in either perdeuterated and amide back-exchanged form (blue) or with algal amino acid labelling (orange). See Fig. S3 (ESI†) for cross sections. (B) Distribution of peak intensities for peaks found in both of the samples and (C) the according relative intensity differences for the algal amino acid-based sample compared to the perdeuterated one (*i.e.* $(I_{algal} - I_{perdeut})/I_{algal}$). The bin "5 a.u." in Fig. 2B corresponds to approximately 20% of the highest peak intensity found in both samples.

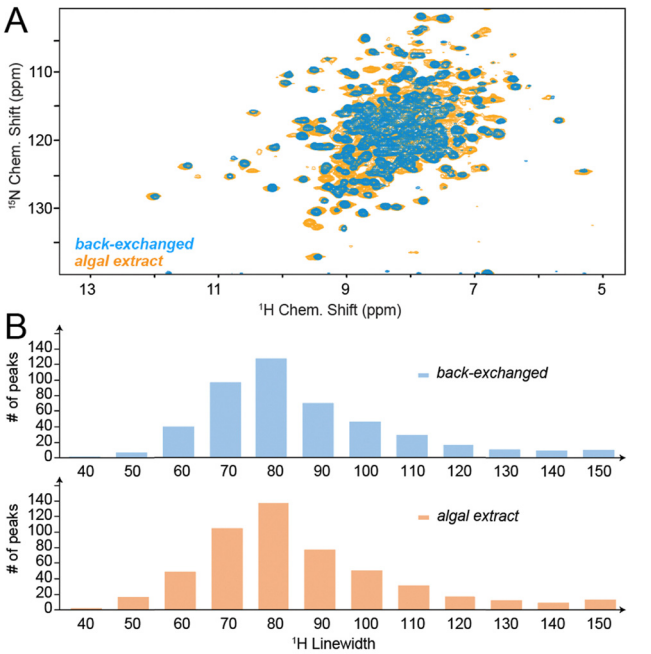

Fig. 3 Resolution and ^{1}H linewidths obtained upon algal amino acid labelling in TS. (A) Overlay of H/N correlations for TS obtained at 700 MHz Larmor frequency from a perdeuterated and ^{1}H-back-exchanged sample (blue) or algal amino acid sample (orange), depicted for an effective proton evolution time of 20 ms and sin^2 apodization with a shift of the bell by $\pi/4$. See Fig. S5 (ESI†) for cross sections. (B) Histograms of proton linewidths obtained for perdeuteration/back-exchange (top) and algal amino acid labelling (bottom), assessed in the 3D hCONH spectra.

suggesting that (in the framework of a six-day hCONH experiment for each of the samples, using a threshold of around 20% relative to the highest-intensity peaks for counting a successful appearance) on the order of 80 additional residues can be found. Importantly, whereas the appearance of many new peaks is obvious, those that are obtained conventionally remain virtually unaltered. To quantitatively address the peak heights of those peaks that are seen in both samples, a correction factor was determined from 1D direct-detection carbon spectra to compensate for slightly differential sample amounts. Fig. S4 (ESI†) shows an overlay of the 1D spectra used for normalization. Fig. 2B shows the normalized peak intensities obtained for the two labelling schemes, whereas Fig. 2C translates the pairwise intensity comparison into a relative intensity difference. Both representations show that the peak intensities of the remaining peaks stay virtually unchanged. A slight increase may be due to peaks that are present even in the perdeuterated samples but have not fully exchanged to 100% protonation upon sample purification.

Given the higher level of deuteration warranted by algal amino acid labelling in the sidechains (Fig. 1D), one would also expect narrow proton linewidths of algal amino acid samples similar to the perdeuterated/back-exchanged reference case. This is indeed the case: Fig. 3A shows a direct overlay of TS H/N correlations processed *via* a standard apodization (sin^2 with shift of the maximum by $\pi/4$). Even though most peaks are heavily overlapped in the 2D, the resolved ones visually suggest the linewidth of the perdeuterated sample to be largely maintained upon algal amino acid labelling (see cross sections in Fig. S5, ESI†). To permit a more quantitative comparison, we assessed the proton line widths obtained within the 3D hCONH spectra (Fig. 3B). The comparison of the obtained histograms suggests a virtually identical linewidth distribution. Whereas we estimate the combined uncertainty in measuring the linewidths and determining the mean of the distribution to amount to at least 5 Hz, it becomes obvious that the use of algal amino acid labelling allows to retain the resolution achieved by perdeuteration even for a sample as extremely well-behaved as micro-crystalline *St.* tryptophan synthase.

Re-using the labelling scheme from Löhr *et al.* for ^{1}H-detected solid-state NMR adds a conceptually straightforward and easy-to-implement strategy to obtain well behaved samples for the highly sought-after 55–65 kHz MAS regime while avoiding refolding for protonation of otherwise inaccessible amide sites. This is of significance in particular for increasingly complex, high-molecular-weight machineries, which have been becoming more and more interesting for recent solid-state NMR studies.[6–9,19] The advantages of circumventing not only the requirement for D_2O-based media but minimal media altogether have been discussed for solution-state NMR applications previously.[12] On the other hand, the cost of an exclusively algal amino acid-based culture, prepared as described in the ESI,† is affordable (see Table S4 with current price estimates in the ESI†). It is known that part of the amino acid types suffer from acid- or base-catalysed hydrolysis, which is usually

involved for commercial production of amino acid mixes.[15,20,21] This likely explains the relatively high remaining protonation levels in Asn and Glu (Fig. 1D). Rigorous use of complete mixes of deuterated amino acids (instead of an algal lysate) might bring the protonation levels of all amino acid types down to the values observed for *e.g.*, Ala, Arg, Lys, and Thr. Solid-state NMR of deuterated proteins, in particular micro-crystalline samples, has the potential to address proteins larger and more complex than those accessible in solution NMR.[7,22] The limitations with respect to the water-inaccessible hydrophobic core has, however, constituted a general, main bottle neck for exploitation of recent technological innovations for high-molecular-weight proteins. Exploiting algal amino acid labelling for current solid-state NMR approaches, while maintaining narrow spectral lines, hence provides exciting opportunities for the study of increasingly complex targets. Even if the most insensitive experiments will be compromised by the partial H^{α} protonation remaining, a breadth of experiments will be well feasible from algal amino acid labelled samples, which can at least complement the spectra obtained from perdeuterated/back-exchanged samples to fill up remaining gaps in assignment and thus enable comprehensive NMR-based characterization.

In conclusion, we have shown that H_2O-based expression media that *exclusively* feature deuterated algal amino acids instead of deuterated glucose or glycerol, while overcoming the barrier of solvent exchange in water-inaccessible protein segments, at the same time largely maintain the spectral properties of perdeuterated samples even under moderate MAS. The labelling scheme already known from solution NMR may help to facilitate comprehensive, high-sensitivity solid-state NMR assessment of deuterated proteins including their hydrophobic core, hence overcoming one of the pertinent bottlenecks for elucidation of increasingly complex biological machineries.

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Conflicts of interest

There are no conflicts to declare.

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