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A simple and inexpensive preparation of perdeuterated sorbitol for use as a biomacromolecule stabilization agent in NMR studies

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Abstract

Sorbitol is an excellent protein-stabilization agent, but it is typically used at high concentrations where the ^{1}H signals can interfere with NMR data collection and analysis. Deuteration of sorbitol can ameliorate this problem; however, perdeuterated sorbitol is not commercially available. We describe a simple and inexpensive method for preparation of perdeuterated sorbitol from perdeuterated glucose. The method is described explicitly and examples are given where the use of perdeuterated sorbitol has allowed the extraction of information, from NMR spectra, that is otherwise unobtainable.

Solution state structural studies of biomacromolecules by NMR methods place stringent demands on the behavior of target molecules under experimental conditions. Biomacromolecules must be soluble to at least several hundred micromolar and non-aggregating. They must also be structurally stable for extended time periods, preferably several months at room temperature. A number of excipients can be used to stabilize folded protein conformations. These include small zwitterionic species such as glycine and its N-methylated derivatives and polyols such as glycerol, sorbitol, and trehalose (Yancey et al., 1982; Santoro et al., 1992; Costantino et al., 1998). These compounds are typically added at high (several hundred millimolar) concentrations, which can interfere with NMR data acquisition (Wimmer et al., 1997).

In our studies of the chromatin modifier (chromo) domain of the fission yeast protein Clr4, we have found that addition of 10% w/v sorbitol extends the lifetime of the folded conformation from less than two weeks to over two months at 25 °C. In our experience, sorbitol is more effective than glycerol for conformational stabilization when used at equivalent w/v ratios. Sorbitol yields a less viscous solution than glycerol, and buffer containing sorbitol is amenable for use in measurement of residual dipolar couplings. We have been able to establish ordered phases in a solution containing 10% w/v sorbitol using the standard protocols for phospholipid bicelle preparations.

At 10% w/v, the sorbitol concentration is 0.55 M, giving a (natural abundance) ^{13}C concentration of 5.5 mM for each of the six carbon atoms. This is often 5–10 times typical protein concentrations. Sorbitol resonances present no acquisition difficulties in $^{1}H^{N}$-detected experiments. However, the large, slowly decaying signals between 3.7 and 4.0 ppm (^{1}H) result in large ridges in the indirect dimensions of $^{1}H^{C}$ detected, ^{13}C-correlated experiments such as the ^{13}C-edited NOESY-HSQC and the HCCH-TOCSY. Our solution to this difficulty is the use of perdeuterated sorbitol, which can be conveniently and economically prepared by reduction of perdeuterated glucose with $NaB^{2}H_{4}$.

Procedures for reduction of aldoses to alditols are well established (Wolfrom and Thompson, 1963). Although our methods follow published procedures, we explicitly describe them here to highlight a sin-

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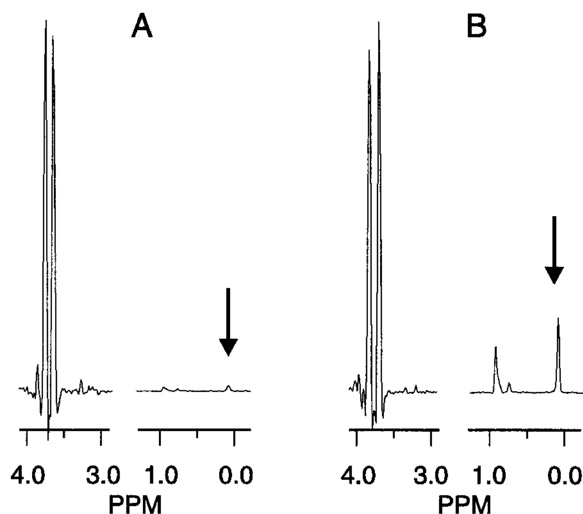

Figure 1. ^{1}H traces through [^{13}C,^{1}H]-HSQC spectra collected on the Clr4 chromo domain (0.9 mM) in (A) ^1H_2O/^{1}H-sorbitol and (B) ^2H_2O/^{2}H-sorbitol. Arrows indicate the ^{1}H resonance of V25 γ_1. Traces through the protein resonance are drawn at the same scale as the sorbitol resonances in each spectrum. Protein resonance to sorbitol resonance peak height ratios are 1:60 with ^{1}H-sorbitol and 1:4.2 with ^{2}H-sorbitol.

gle pathway suitable for use in a structural biology-oriented laboratory. One gram of 1,2,3,4,5,6′,6″-^{2}H glucose (98% enriched, purchased from C.I.L., Andover, MA) is dissolved in 10 ml ^2H_2O in a 500 ml round-bottom flask. To this is added, drop-wise, 0.5 g of NaB^2H_4 (Sigma, St. Louis, MO), previously dissolved in 10 ml ^2H_2O. (This amount of NaB^2H_4 represents a substantial molar excess for 1 g sorbitol. Less borodeuteride can be used, albeit with a lengthening of the reaction time.) The reaction mixture is stirred, at room temperature, until assays for glucose are negative (total reaction time is typically 30–60 min). Completeness of the reaction can be monitored by standard assays for glucose. We have found two methods particularly convenient: (1) Several μl of the reaction mixture is acidified (to oxidize excess borodeuteride), added to 200 μl Benedict's solution (Furniss et al., 1989), and boiled for several minutes. This will produce a reddish precipitate in the presence of an oxidizing agent (glucose). (2) Several μl of the reaction mixture is acidified, neutralized, and applied to standard blood/urine glucose detection strips. We have used Diastix (Bayer, Elkhart, IN), which are inexpensive, readily available and convenient. This assay involves glucose oxidase and horseradish peroxidase, and it is important to neutralize the reaction mixture following acidification, to avoid denaturation of the enzymes. When the reduction is complete, Na^+ ions are scavenged by addition of an excess of a cation exchange resin, such as Amberlite IR-120(plus) (Sigma). This results in a strongly acidic solution, which also oxidizes the remaining borodeuteride. The resin is removed by filtration.

Borate ions form tight complexes with sorbitol, and recrystallization of sorbitol directly without prior removal of borate does not appear feasible (Chaikin and Brown, 1949). Several methods for removal of borate from alditols have been described. These include (1) ion exchange chromatography, (2) addition of methanol and evaporation of methyl-borate complexes, and (3) acetylation, recrystallization, and deacetylation (Lewis et al., 1963). We have found that sorbitol adheres to many anion exchange resins and that the high borate concentration in the reaction mixture interferes with acetylation, due to the tendency of the sorbitol–borate mixture to form a hydrated glass when dried. The second method described above fulfills our requirement for a simple, high yield procedure by which we can obtain purified sorbitol.

The acidified reduction mixture is evaporated at reduced pressure and redissolved in 100 ml methanol with 5 ml acetic acid. This solution is repeatedly evaporated and redissolved in methanol/acetic acid to evaporate the borate ion as a volatile methyl borate compound. In our experience, at least several hundred milliliters of methanol is required to remove all of the borate. Following removal of all borate, as measured by the carmine assay (Hatcher and Wilcox, 1950), the solution is evaporated to a syrup under reduced pressure and recrystallized from 5 ml methanol at −20 °C. The yield is typically over 60%. ^{13}C-observed NMR spectra of the product confirmed its identity as sorbitol and the retention of $\geq$98% deuteration. To remove residual methanol that is trapped in the crystal lattice, the sorbitol crystals should be redissolved in and lyophilized from ^2H_2O several times. Because of the hygroscopic nature of sorbitol, it can be difficult to obtain absolutely dry material. However, the concentration of sorbitol in aqueous solution can be easily measured by a colorimetric assay with $CuSO_4$ and NH_4OH (Grimble et al., 1983). Perdeuterated sorbitol can be added to an NMR sample directly, in which case only 20–30 mg is needed for a 10% w/v solution. For samples that cannot be successfully concentrated in the absence of sorbitol, an 850 μl Sephadex G25 desalting column (Amersham-Pharmacia, Piscataway, NJ), equilibrated with 1 ml ^{2}H-sorbitol-containing buffer, will allow nearly complete exchange. For a

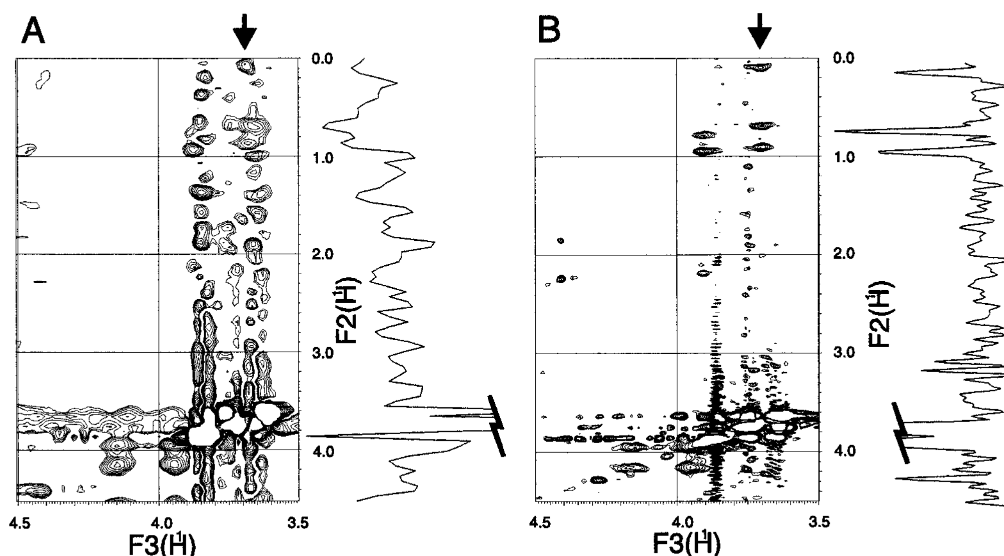

Figure 2. ^{1}H-^{1}H planes (^{13}C shift = 64.7 ppm) from ^{13}C-edited 3D-NOESY-HSQC spectra acquired in (A) ^1H_2O/^{1}H-sorbitol and (B) ^2H_2O/^{2}H-sorbitol. 1D traces are taken along F3 = 3.7 ppm (^{1}H, acquisition) as indicated by arrows. Cross peaks that are obscured by F2 ridges in ^{1}H-sorbitol-containing buffer are clearly observable in spectra obtained from ^{2}H-sorbitol-containing buffer. The protein concentration in each case is ~0.9 mM.

10% w/v solution, this method requires approximately 100 mg ^{2}H-sorbitol.

Figure 1A shows one-dimensional traces through [^{13}C,^{1}H]-HSQC spectra collected on a ^{13}C,^{15}N-labeled sample of the Clr4 chromo domain in ^1H_2O, ^{1}H-sorbitol. Sorbitol and protein resonances are shown to scale. Figure 1B shows the analogous traces following exchange of ^{1}H-sorbitol for ^{2}H-sorbitol using a Sephadex G25 desalting column. The decrease in intensity of the sorbitol resonances relative to the intensity of the protein resonances is approximately a factor of 12. The difference between the actual reduction in sorbitol resonance intensity and the predicted 98-fold reduction (assuming 98% ^{2}H-enriched glucose as starting material) is caused by the use of the desalting column as the method of sorbitol exchange, and not a reduction in deuterium enrichment of the sorbitol (vide supra). Use of a larger desalting column will give more complete exchange, but at the expense of requiring more perdeuterated sorbitol.

A 10- to 15-fold reduction in (^{1}H) sorbitol intensity is sufficient to allow data collection in many cases. Figure 2 shows ^{1}H-^{1}H planes from a three-dimensional, ^{13}C-edited NOESY-HSQC spectrum. The spectrum shown in Figure 2A was acquired in ^1H_2O, ^{1}H-sorbitol, while the spectrum shown in Figure 2B was acquired in ^2H_2O, ^{2}H-sorbitol, prepared as described above. As is evident from the one-dimensional traces, spectra collected in ^{2}H-sorbitol-containing solution allow detection and quantitation of NOEs which are otherwise obscured by ridges emanating from the sorbitol protons in spectra collected in ^{1}H-sorbitol-containing solution. We also note that reduction in sorbitol resonance intensity can be optimized during INEPT transfer by placing large gradients centered as far from the refocusing 180° pulses as possible to take advantage of the faster diffusion of sorbitol relative to proteins.

Sorbitol, at concentrations up to at least 10%, is also compatible with the formation of stable, liquid crystalline solutions suitable for measurement of residual dipolar couplings (Tjandra and Bax, 1997). We have successfuly obtained ordered phases in a 4.2% (w/v) DTPC:DHPC (3:1, mol:mol) solution containing 10% sorbitol. Hence, measurement of the residual dipolar couplings by experiments which detect $^1H^{\alpha}$, such as the $^1H^{\alpha}$-coupled HCA(CO)N and $^{13}C'$-coupled, $^1H^{\alpha}$-decoupled ^{1}H-^{13}C HSQC (Bewley et al., 1998), is feasible even in the presence of significant concentrations of sorbitol.

In conclusion, we have demonstrated that the method of borohydride reduction of aldoses to alditols provides a simple and inexpensive method for the preparation of perdeuterated sorbitol. Preparation of perdeuterated sorbitol does not require hazardous materials (beyond sodium borodeuteride) or reactions and

can be accomplished in several days with equipment commonly available in structural biology laboratories. The yield is such that 1 g of perdeuterated glucose will produce sufficient perdeuterated sorbitol for use in 5–10 samples. Hence the cost per sample is quite reasonable. Perdeuteration of sorbitol allows for its use at high concentrations as a protein-stabilization agent for NMR studies without causing substantial interference with normal data acquisition.

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