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Deuteration of human carbonic anhydrase for neutron crystallography: Cell culture media, protein thermostability, and crystallization behavior

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ABSTRACT

Deuterated proteins and other bio-derived molecules are important for NMR spectroscopy, neutron reflectometry, small angle neutron scattering, and neutron protein crystallography. In the current study we optimized expression media and cell culture conditions to produce high levels of 3 different deuterated human carbonic anhydrases (hCAs). The labeled hCAs were then characterized and tested for deuterium incorporation by mass spectrometry, temperature stability, and propensity to crystallize. The results show that is possible to get very good yields (> 10 mg of pure protein per liter of cell culture under deuterated conditions) and that protein solubility is unaffected at the crystallization concentrations tested. Using unlabeled carbon source and recycled heavy water, we were able to get 65–77% deuterium incorporation, sufficient for most neutron-based techniques, and in a very cost-effective way. For most deuterated proteins characterized in the literature, the solubility and thermal stability is reduced. The data reported here is consistent with these observations and it was clear that there are measurable differences between hydrogenous and deuterated versions of the same protein in T_m and how they crystallize.

1. Background

1.1. Protein deuteration for neutron protein crystallography

Hydrogen atoms (^{1}H, H in this manuscript) are abundant in biological macromolecules and in proteins constitute ~50% of the total number of atoms. H atoms are important in structural biology as they are involved with many aspects of protein structure and function. H atoms are integral to hydrogen bonding (H-bonding) interactions, solvent networks, enzyme catalytic mechanisms, and ligand binding [27]. In spite of their importance, they are challenging to observe with standard techniques such as X-ray crystallography, and their positions are often inferred, assumed, or ignored. A large part of the difficulty in observing H atoms directly is that they have one electron and the magnitude of X-ray scattering depends on the atomic Z number of an element. Neutrons are a very useful and complementary probe as neutron scattering occurs from atomic nuclei and the magnitude is independent of the number of electrons (www.ncnr.nist.gov/resources/n-lengths/accessed 26/02/2018). In practical terms, this means that it is possible to observe light atoms as readily as the heavier atoms, C, N, and O. Neutrons are able to discriminate between different isotopes of the same element, and the discrimination is particularly good for ^{1}H and its isotope ^{2}H (deuterium, D in this manuscript), with scattering lengths of −3.74 fm and +6.67 fm, respectively. The negative scattering of H contributes to signal cancellation of positive scattering neighboring atoms. In addition to the negative scattering of H, it also has intrinsically a large incoherent scattering cross-section (~80 barn) that leads to high background on neutron detectors, effectively reducing the signal-to-noise ratio. Taken together, it is greatly advantageous to deuterate proteins for neutron protein crystallography (NPX) measurements [3,19]. In this manuscript “deuterate” refers to partial deuteration that includes H/D exchange, while perdeuterate refers to full deuteration (~99% D incorporation).

An informal survey of the Protein Data Bank (https://www.rcsb.org/pdb/home/home.do accessed 11/11/2017), revealed that for the ~125 available neutron crystal structures, deuteration for NPX involve three different approaches: 1) deuteration through H/D exchange (only labile H are replaced with D); 2) deuteration through expression of protein in D_2O-containing media but using an unlabeled (hydrogenous) carbon source; 3) perdeuteration through expression of the protein under deuterated conditions using a perdeuterated carbon source. For the neutron crystal structures reported in the PDB, deuterated proteins

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were either obtained commercially or through recombinant expression in *E. coli* using minimal media. The majority of reported neutron crystal structures have used approaches (1) and (2), but the number of per-deuterated protein crystal structures has increased in the last ~5 years [3,26]. Deuteration offers significant benefits in neutron diffraction data collection as the signal-to-noise ratio is improved, incoherent background is reduced, and deuteration enables the use of smaller crystals [3].

The use of heavy water (D_2O) and D-labeled carbon source, whether glycerol or glucose, makes it very expensive to produce fully deuterated (perdeuterated, > 99% D incorporation) labeled proteins for neutron experiments (SI, Table S2). Using fresh D_2O *or* labeled glycerol leads to a 2-fold increase over the approach described in this work. If both fresh D_2O *and* labeled glycerol is used, the cost increases 4-fold (Table S2, calculation shown in Supplemental Information). In addition, protein expression yields tend to be lower under deuterated conditions and, as such, there is an incentive to find cost-effective ways to deuterate proteins, while optimizing the level of D incorporation [24,28]. Due to the cost and effort involved, often H/D exchange is considered "good enough" for most experiments and the fact that the majority of neutron structures are determined from such samples support this [3]. The choice of partial deuteration vs. perdeuteration heavily depends on what the researcher is interested in seeing. For well-hydrated enzyme active sites with polar or charged amino acid residues, H/D exchange is usually rapid and sufficient enough to enable visualization of D atoms on the protein and solvent and allows detailed structural investigation of H-bonds, ligand binding and so on [26]. However, due to the uncertainty of partial H/D exchange, the data (nuclear density maps) can often be ambiguous and difficult to interpret. Hydrophobic residues with non-labile H atoms can display significant signal cancellation, leading to "gaps" in the maps. The most ambiguous situation is where partial occupancy of H and D at the same position is possible (e.g. His and Lys side chains). Here a combination of ~70% H and ~30% D can lead to total cancellation, leaving the researcher with no useful data on the charged state of such residues [10]. So, even while the yields are poor and the cost high, it is advantageous to deuterate or perdeuterate proteins for NPX experiments where possible. For small angle neutron scattering (SANS) and neutron reflectometry (NR) the motivation to D-label is different, and depends on the system under study. Unlike crystallography where D-labeling is desirable to reduce the incoherent background and to enable direct visualization of individual H/D atoms, for SANS and NR the most important application of biodeuteration is to enable contrast matching for studies on multi-component (e.g. protein-protein) systems. Here researchers aim to produce a labeled protein with a level of D incorporation so that SANS measurements in different ratios of D_2O/H_2O selectively mask out (make invisible) the labeled protein over the unlabeled protein [37]. A recent report looked at yeast and bacterial expression systems, using unlabeled carbon sources, to produce partially labeled proteins with 65–70% D incorporation. Investigating these proteins with SANS showed that this level yielded a contrast match-point of 97–99% D_2O [9]. This level of deuteration allows for measurements at 0, 40, and ~100% D_2O solvent contrasts and yields structural information on labeled and unlabeled components in complexes.

1.2. Human carbonic anhydrases

In humans there are 15 expressed carbonic anhydrases (hCAs) with different subcellular distribution, which can be cytosolic, membrane bound, or secreted. HCAs are present in a variety of tissues and are involved with a wide range of physiological processes. HCAs catalyze the interconversion of CO_2 to HCO_3^-, with a subsequent proton transfer (PT) step that completes the catalytic cycle [34]. One of these isoforms, hCA IX, is not usually expressed in healthy tissues but is found in over 30 kinds of solid tumor cancers to date. This makes it an obvious cancer treatment and imaging target [1]. Due to the high sequence and structural conservation between hCAs, there exist significant challenges in side-effects due to off-target binding [21,29]. We are interested in neutron and X-ray studies of different isoforms of hCAs to map H-bonding in the active site, the protonation state of active site residues, the details of water networks in and out of the active site, and to investigate inhibitor binding interactions to the enzyme's active site [11]. To facilitate neutron studies there is a need for deuterated hCAs in sufficient quantities to allow for crystallization and scale-up to obtain large (0.5–1.0 mm^3) single crystals for neutron data collection.

Previous studies have investigated the X-ray crystal structure of perdeuterated hCA II isoform and found there to be no change in the protein structure or solvent arrangement compared to crystal structures of hydrogenous versions [5]. Enzymatic assays with hCA II were carried out to investigate isotope effects on the kinetic parameters of hCA II and the results, while complex, indicate different isotope effects for pH-dependent and pH-independent parameters [35]. Steady-state kinetic parameters showed a significant isotope effect (~4 fold lower), when comparing the k_{cat}^{h} and k_{cat}^{d} for the CO_2 and HCO_3^- interconversion reaction. Interestingly, the opposite was observed for the non-physiological activity of hCA II, the esterase activity. In assays with *p*-nitrophenyl acetate the enzyme was ~35% faster in D_2O compared to H_2O [35]. These previous studies showed that deuteration of hCA II did not affect the crystal structure and that the presence of D_2O slowed down catalysis, but did not change how it proceeds. These are important factors to consider when embarking on neutron experiments and it is important to show that the system under study is relevant to the hydrogenous, physiological version.

For the current study we set out to compare methods to make deuterated hCAs, determine the level of D incorporation by MS, and to characterize the protein stability and crystallizability. Ultimately we would like to use them for neutron crystallographic studies and the results represent an enhancement over our current approach of using H/D exchanged proteins that contain only 25–30% exchanged D atoms in labile positions. We compared different cell culture media types commonly used for D-labeled protein production and were able to get very good yields using unlabeled glycerol. The thermal unfolding temperatures were measured to compare H- *vs.* D-protein properties, as a function of both pH and H_2O/D_2O. Finally, side-by-side crystallization trials were set-up to observe whether the deuterated hCAs crystallized as well the H counterparts.

2. Materials and methods

2.1. Deuterated and hydrogenous protein expression

For this study we worked with three different enzymes of the human carbonic anhydrase family [39]. The transcripts used were as follows: 1) Wild type hCA II (WT hCA II), 2) hCA IX mimic, 3) hCA IX surface variant (SV). All three are cloned into commercial pET vectors without a His-tag. For WT hCA II we used an expression plasmid based on pET31F1 [36]. The cDNA encoding hCA IX mimic and hCA IX SV was synthesized with codon optimization for expression in *E. coli* and purchased from GenScript. The hCA IX mimic and hCA IX SV coding region were cloned into pET-26b (+) (Novagen) using NdeI/XhoI. HCA IX mimic was originally designed by Genis et al. and developed further by Pinard et al. via site-directed mutagenesis of the active site of wild type hCA II which gives a structural analogue to the native hCA IX catalytic domain while keeping the overall physical attributes of hCA II which is soluble, easy to express, and crystallizable [14,29]. The hCA IX surface variant was described previously [21]. Briefly, it is a wild-type version of the soluble catalytic hCA IX domain that was engineered to have 6 surface mutations that provide more soluble and stable enzyme [21].

WT hCA II was expressed in *E. coli* BL21 (DE3) cells under ampicillin selection. HCA IX mimic and hCA IX SV were expressed in BL21 (DE3) cells under kanamycin selection. For both H and D proteins the overnight starter cultures were grown in Luria Broth (LB) media with