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Protein Perdeuteration for Neutron Crystallography

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Abstract.

Protein deuteration by recombinant expression in deuterated minimal media offers advantages to several experimental techniques involving neutron scattering. Perdeuteration improves neutron crystallographic studies of proteins. Although not required, protein perdeuteration offers advantages regarding crystal size, data collection time, and the clarity of the resulting experimental nuclear density maps. The level of advantage of perdeuteration depends on practical matters of the existing method of protein production. If recombinant expression yields are high, and purification is efficient, the staff and consumable costs for protein perdeuration are not so great.

KEYWORDS: Deuteration, perdeuteration, protein expression, neutron crystallography

1. Introduction

Protein deuteration is the production of protein incorporating the hydrogen isotope deuterium (D, or H-2) in place of the prevalent hydrogen isotope, H-1. The usual method for biological deuteration is by recombinant expression in a deuterated minimal medium, whereby the amino acids are fully synthesized from the components of the medium.[1] A high level of D_2O as the solvent results in a high level of protein deuteration. Use of a deuterated carbon source and 100% D_2O results in a perdeuterated protein.

Protein perdeuteration, in current practice, means a deuteration level of ~99 %. The prefix “per” comes from Latin with the meaning “very”, and means a deuteration to a level as high as practically reasonable. The practical limits are determined by the suppliers of the deuterated consumables. D_2O is usually available commercially containing ~99.8% D, deuterated glycerol or glucose at ~98% D, and combined these produce a perdeuterated protein, where ~99% of the non-exchangeable hydrogen atoms are deuterium, D.

The absence/presence of the neutron in the nucleus of the two hydrogen nuclei affect neutron scattering. H-1 has a negative neutron scattering length density (nSLD), of about

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half the magnitude of the nSLD of H-2. Carbon, nitrogen and oxygen have similar nSLD values to H-2 (see Figure 1). With hydrogen atoms comprising approximately half the number of atoms in a protein, swapping H-1 for H-2 enables convenient and useful nSLD-labelling strategies for multi-protein structural determinations[2]. Protein-specific deuterium labelling enables clever experiments to be done on multi-protein complexes by methods such as small angle neutron scattering (SANS). A recent example of the value of deuteration and SANS for protein structural determination was the study of large domain disorder-to-order transition upon the interaction of ScsB and ScsC, proteins enabling copper-resistance of the human pathogens, *Proteus mirabilis* [3]. Protein deuteration is also very important for neutron reflectometry (NR), neutron backscattering, and nuclear magnetic resonance (NMR). For neutron crystallography of proteins, deuteration is a complicated question, and is the topic of discussion of this paper.

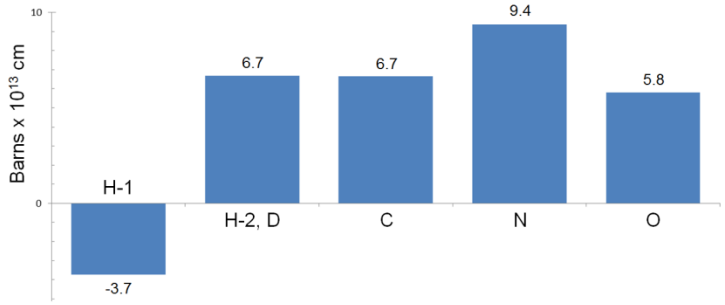

Figure 1. Coherent neutron scattering lengths. H-1 has a neutron scattering length density (nSLD) of opposite sign and approximately half the magnitude of carbon. H-2, or D, has a very similar nSLD to carbon. Nitrogen and oxygen have similar positive nSLDs. Not shown are incoherent scattering lengths densities. H-1 has a very large incoherent nSLD, while the other nuclei have small to negligible incoherent nSLDs. Incoherent nSLD produces isotropic signal that serves helpful purpose and contributes noise. Data taken from Engelman and Moore, 1972 [2].

Neutron crystallography of proteins offers advantages to structural studies of proteins, as an extension of x-ray crystallography. The main advantage is the ability to definitively observe structured hydrogen atoms. Another advantage is the avoidance of photoreductive damage to the reactive centres, common in x-ray crystallography[4]. These advantages are necessary for some ambiguous chemical questions of catalytic mechanisms.

An example of the productive use of neutron crystallography is the study of the catalytic centre of cholesterol oxidase, where a long N-H proton was observed. This polarised amide, nearly contacting the catalytic nitrogen atom of the cofactor, revealed how the network of electrostatics primes of the catalytic centre. [5]. Another example being studied by neutron crystallography is the catalytic mechanism of rubisco. In its large compact active site, protons may be modelled, but only neutron crystallography will give experimental evidence for the presence and location of the individual protons, and knowledge of these protons is critical to confidence in any computational analysis. Rubisco is a very important environmental player, and considerable efforts have been devoted to improving the catalytic efficiency of rubisco, so far without success. To better understand the link between CO_2 and O_2 reactivity and the rationale of Rubisco diversification and evolution, a better description of Rubisco mechanism is still required.[6]

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In recent years, advances in instrumentation, computational refinement, and availability of beamlines, has greatly expanded access to neutron crystallography, and its use is expanding.[7]

2. Benefits of perdeuteration to neutron crystallography

Deuteration is not required for neutron crystallography. Many significant and well cited neutron protein structures have not involved protein perdeuteration. However, there are two distinct advantages of perdeuterating the protein for neutron crystallography.

The first advantage is the reduction of scattering noise from the incoherent neutron scattering of hydrogen (H-1) nuclei. The reduction or removal of this noise manifests as increased signal-to-noise of the diffraction data[8]. The advantage can be utilised by allowing the use of a smaller crystal[9], and this can be critical, if only small crystals can be grown. Crystal sizes being equal, the perdeuterated protein crystal requires less beamtime to obtain the same quality of data.

The second advantage is of the presentation quality of the results[10]. Using perdeuterated proteins avoids the smearing of negative nuclear scattering density of H-1 atoms into neighbouring positively nuclear scatter atoms, usually C-12 atoms. This is particularly significant for CH_2 groups, where the negative density of the two H-1 atoms nearly cancels the positive density of the one C-12 atom. For data of moderate resolution, undeuterated protein will produce feature discontinuous density along the molecule. An illustration of this effect is shown in figure 2.

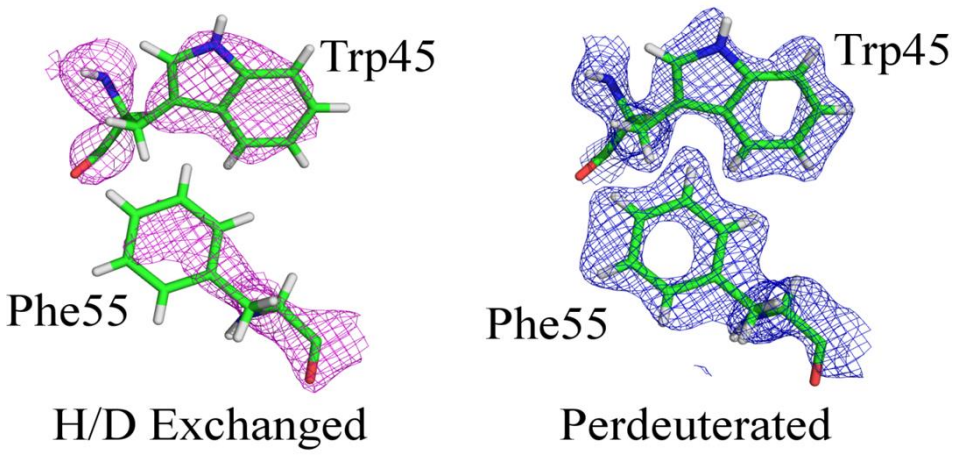

Figure 2. Nuclear density maps, 2.1Å resolution, of H/D exchanged (left) and perdeuterated (right) cholesterol oxidase (Golden, Duff, Meilleur, Vrielink, unpublished figure)
Non-exchangeable hydrogen atoms bonded to positively scattering carbon atoms results in cancellation of density as can be seen on Phe55 and Trp45 of the H/D exchanged structure. Density cancellation is near-complete for CH_2 groups. Perdeuteration circumvents this problem, resulting in continuous intuitive density.

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3. Practicalities, delays, and consumable costs

Perdeuterated protein expression can be difficult, but a method for robust and reliable perdeuteration has been established and described [1]. Assuming experienced staff, a stable protein, and consistently good recombinant bacterial expression yields, the standard perdeuteration method may very well work on first attempt. In such cases, the perdeuteration process may take very little more time than unlabelled production.

However, if the expression method suffers mistakes borne from inexperience, or if the protein is unstable in expression, or doesn't work with the standard recombinant expression method, or for some other reason the perdeuterated production method requires optimisation, there can be risk of severe delay in the production of protein.

If, between performing an x-ray crystallographic analysis and beginning a neutron crystallographic analysis, the commencement of perdeuteration requires a modification of expression method, the possibility of complication is high. Any alteration in sequence, or post-translational modification, or other modification to the expression method may impact the purification method. Any change in protein preparation may impact crystallisation. All of these possibilities present risks to the timeliness of production of perdeuterated crystals.

Protein perdeuteration requires deuterated water (D_2O) and a deuterated carbon source. The deuterated carbon source, whether d-glycerol or d-glucose, is the dominant consumable cost. D_2O may be used very efficiently using a bioreactor to achieve high culture density, but carbon source consumption is directly proportional to biomass production. There are two parameters that vary greatly from protein to protein: expression level, and purification efficiency. A protein that expresses poorly, and purifies inefficiently, will be much more expensive to produce per milligram than another well expressing and easily purified protein. A third variable is the amount of protein required to complete protein crystallisation to achieve crystals of the required size. Finally, the cost of commercially sold deuterated carbon sources is variable. Nevertheless, it is important to estimate costs.

To produce neutron diffraction quality crystals, using parallel crystallisation trays, with some degree of variation of crystallisation conditions, combined with cycles of macroseeding using the same batch of purified protein, means that one should start with approximately 100 milligrams of purified protein. This may be more than enough to complete the project, but it is not wise to begin with a small batch because a later batch can very easily have a difference that affects crystallisation. Changes to crystallisation, even small re-optimisation of conditions during the macroseeding cycles, may alter crystal packing and produce packing heterogeneity in the crystal. Heterogeneity in the crystal will severely reduce the diffraction data quality.

To produce 100 mg of purified protein, if well expressing and efficiently purified, requires about 100 g of carbon source. The recommended choice of carbon source is

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deuterated glycerol. Deuterated glucose is similarly priced, but high glucose is inhibitory to bacterial growth and needs to be added progressively. Seeking to reduce costs by using a simpler labelled substrate, such as d-acetate, or d-succinate, involves significant complications to methodology, impacting both reliabilities and yields, and is not recommended. The cost and supply reliability of deuterated glycerol has historically been subject to large variations. List prices for small quantities can be very different to bulk price. Delivered d-glycerol has varied from 3000 to 7000 JPY per gram. Multiplying these factors together gives an estimate for consumables for protein deuteration of sufficient quantity to start a neutron crystallography project of 300 000 to 700 000 JPY.

4. Conclusion

Perdeuteration is not required for neutron crystallography. In some circumstances, you will not want to perdeuterate the protein. Example circumstances would include: already-existing near-millimetre well-diffracting crystals; or if protein is derived from a natural source; or the need for a neutron crystallographic characterisation is urgent and unlabelled purified protein is at hand and beamtime is not limited.
In other cases, perdeuteration will be desirable. For a pre-planned project, the perdeutertion additional expense is moderate. It is moderate compared to an estimate of cost of neutron diffraction beamtime, and it is very small compared to the cost of staff time. The experimental nuclear-density maps with be intuitive.

5. Acknowledgment

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6. Conflict of Interest Statement

The National Deuteration Facility, ANSTO Australia, provides protein perdeuteration services.

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