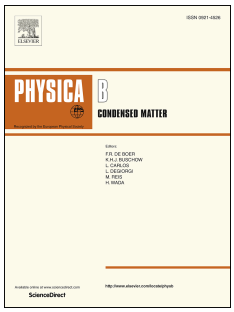
Accepted Manuscript



Deuterium effects on human serum albumin in solution

John White, Valerie Laux, Nick Fraser, David Heß, Michael Haertlein, Trevor Forsyth

PII: S0921-4526(18)30095-4

DOI: 10.1016/j.physb.2018.01.065

Reference: PHYSB 310712

To appear in: *Physica B: Physics of Condensed Matter*

Received Date: 4 August 2017

Revised Date: 12 January 2018

Accepted Date: 31 January 2018

Please cite this article as: J. White, V. Laux, N. Fraser, D. Heß, M. Haertlein, T. Forsyth, Deuterium effects on human serum albumin in solution, *Physica B: Physics of Condensed Matter* (2018), doi: 10.1016/j.physb.2018.01.065.

PHYSICA B paper

Deuterium Effects on Human Serum Albumin in Solution.

John White*, Valerie Laux‡, Nick Fraser*, David Heß‡, Michael Haertlein‡, Trevor Forsyth‡†,

*Research School of Chemistry, Australian National University
† Faculty of Natural Sciences, Keele University, Staffordshire, ST5 5BG, UK
‡Institut Laue Langevin, Grenoble , France
Partnership for Soft Condensed Matter, ILL

1.0 Introduction

We report the thermodynamic effects of heavy water and intramolecular deuteration on the melting and aggregation of fatted and defatted human serum albumin (FHSA and DFHSA) in 250mM salt. Eight different combinations with and without deuterium were produced by recombinant expression of the proteins in the yeast *Pichia pastoris.* Human serum albumin is 47% of the protein in blood and is an important carrier of fat and medicines throughout the body. It is a potential carrier of invasive nanoparticles. The x-ray crystal structures of the molecule in its native and recombinant forms, with and without the fat molecule, and with drug molecules have been determined to 1.8Å resolution by x-ray diffraction [1,2,3]. The structures of the fatted and defatted molecules in this 67kD molecule are very similar and the local conformational changes associated with the fat ligand as well as drug molecules are established.

It is well known that the structures of fully deuterated proteins and their hydrogenous counterparts are closely similar [4,5,6,7] and so it was a surprise, in 2013, that strong aggregation of a fully deuterated sample of human serum albumin in heavy water buffer was found at the outset of a neutron experiment to study the protein corona around silica nanoparticles.

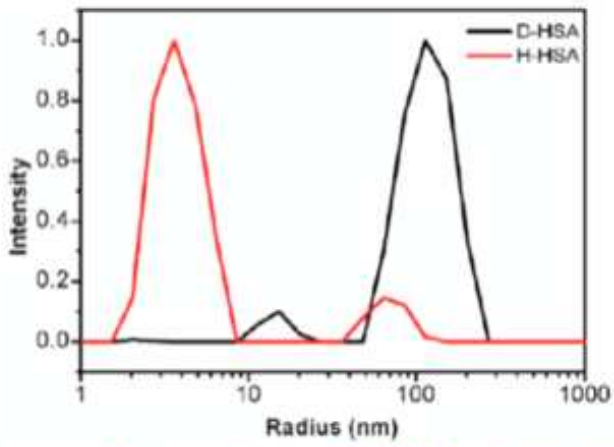


Figure 1 shows the Dynamic Light Scattering at 25°C in heavy water PBS from a 1mg/ml solution of "native" fatted human serum albumin (red) and a 1mg/ml heavy water solution of recombinant fully deuterated, defatted human serum albumin (black).

2.1 Methods

To understand this problem the relatively insensitivity of the native forms to aggregation in D_2O was established [8]. Subsequently in 2014, eight recombinant per-deutero and proto FHSA and DFHSA in 150mM salt tris buffer showed significant and varied aggregation in a period of about a week at about 25°C. Furthermore, the extent of aggregation was dependent on the placement of the deuterium [9]. In 2016 the whole experiment was repeated with highly stabilised HSAs using; the recombinant expression developed by Belew et al (10) in high salt (250mM). During the synthesis 10mM sodium caprylate [11,12] was added for stabilisation and added cysteine (10mM) to block any association by the sulf-hydril linkages. In a parallel synthesis the fully hydrogenous protein was expressed and parts of both recombinant protein solutions were defatted as different deuterium effects were expected for FHSA and DFHSA because of different reactivity [13] and isoelectric points (FHSA 4.8 and DFHSA 5.6 [14]. The synthesis is set out in Figure 2 with the final light or heavy water buffer containing 250mM NaCl, 1mM DTT and 0.1M potassium phosphate.

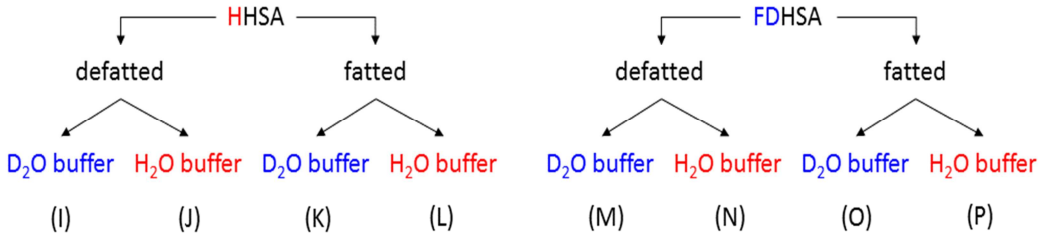


Figure 2 The two syntheses and their Fatted and Defatted human serum albumin products.

The in solution melting and aggregation in high salt (250mM) of these compounds as determined by differential scanning fluorometry using a Nanotemper Prometheus NT.48. The "NanoTemper" method [15] is presented here to quantify the variable effects of solvent and intra-molecular deuteration found previously. The permutation of deuterium labelling is listed in Table 1. The pH is as measured on the meter and the 0.4 pH units to show the real acidity, have not been added [16].

TABLE 1
Recombinant Human Serum albumins, concentrations, pH

Concentration	mg/ml	pH
I - HHSA Defatted D_2O Buffer	1.1	6.74
J - HHSA Defatted H_2O Buffer	1	7.18
K - HHSA Fatted D_2O buffer	1.1	6.74
L - HHSA Fatted H_2O Buffer	1	7.18
M - FDHSA Defatted D_2O Buffer	1.2	6.74
N - FDHSA Defatted H_2O Buffer	1	7.18
O - FDHSA Fatted D_2O Buffer	1.1	6.74
P - FDHSA Fatted H_2O Buffer	1	7.18

3.0 Results

Protein Melting in solution

The form of the NanoTemper melting curves from the instrument and their first derivatives are shown in Figure 3. The 250mM salt solutions provide thermodynamic data for the eight recombinant and the native fatted (Sigma-Aldrich A8763) and defatted (Sigma-Aldrich A1887) proteins – used as a comparison for the effect of fat. The data were recorded between 20°C and 95°C at a scanning rate of 1°C per minute. The results are shown in Table 2.

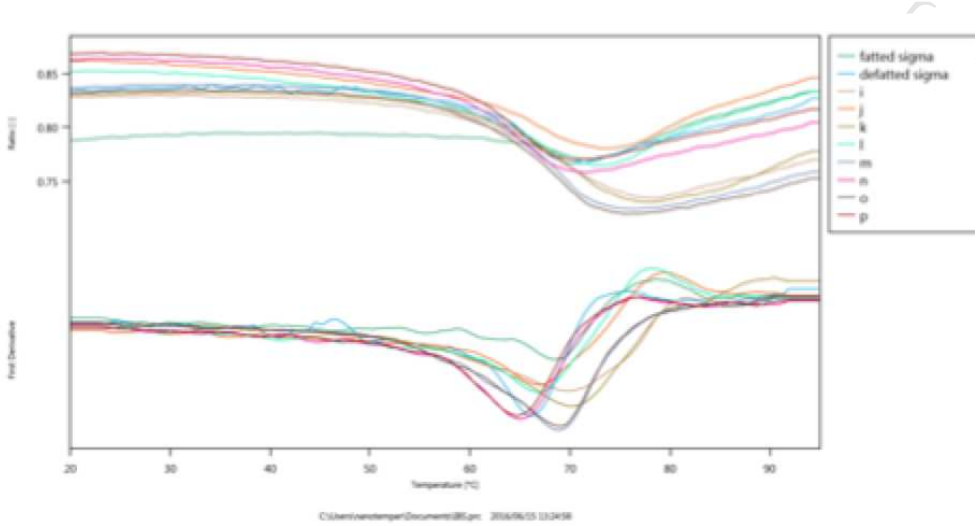


Figure 3 Melting curves F350/330 **fluorescence intensity ratios (upper) and the differential (below) to show the melting temperature and onset.**

TABLE 2
Melting Temperatures for eight recombinant human serum albumins

	From IGOR replots	
	T_1/°C	T_2/°C
Native Fatted HSA	68.7	
Native Defatted HSA	66.2	
I - HHSA Defatted D_2O Buffer	70.3	87
K - HHSA Fatted D_2O buffer	71.0	82
J - HHSA Defatted H_2O Buffer	66.8	87
L - HHSA Fatted H_2O Buffer	66.9	85
M - FDHSA Defatted D_2O Buffer	69.2	-
O - FDHSA Fatted D_2O Buffer	68.9	86
N - FDHSA Defatted H_2O Buffer	64.9	90
P- FDHSA Fatted H_2O Buffer	64.9	89

The expected stabilisation [11,12] by the fatty acid ligand of about 3°C for the native forms appears with a T_1 of 68.7°C for the fatted and 66.2°C for the defatted HSA.. The recombinant data fall into four groups of two proteins each, i.e. I/K, J/L, M/O and N/P. The similarity of the melting function within these groups is striking as is the absence of the 3°C shift from fatted to defatted change – an explanation for which might be that the defatting step in the synthesis may not have been successful.

Changing from light to heavy water buffer increases $T_1$by 3.3°C and 3.5°C for samples I/K vs. J/L and M/O vs. N/P, respectively. Intramolecular deuteration on the other hand decreases the unfolding temperature by 1.9°C and 2.9°C for samples J/L vs. N/P and I/K vs. M/O, respectively.

Thermodynamics

The melting points, T_1, of I…P solutions are 3°C or 4°C higher in D_2O compared to H_2O for both the fatted and "defatted" molecules. and the highest T_1 of all - for the deuterated molecule, shows that there is a solvent and an intra-molecular deuterium effect present - about (+3°C rise for the solvent and an extra 1°C attributable to molecular deuteration.

The van't Hoff enthalpies and entropies, ΔH and ΔS, of melting can be calculated in principle, from the fluorescence intensity ratios of the melting curves, F350/330, as a function of temperature [17,18,19]. To obtain the van't Hoff curves, the as-measured fluorescence ratio from each sample, normalised to unity and then the fractional fluorescence ratio at any given temperature subtracted from unity and ln plotted – versus the reciprocal of absolute temperature (Figure 4) and the straight-line portion used. In fact the noisiness of the data makes the choice of background very difficult.

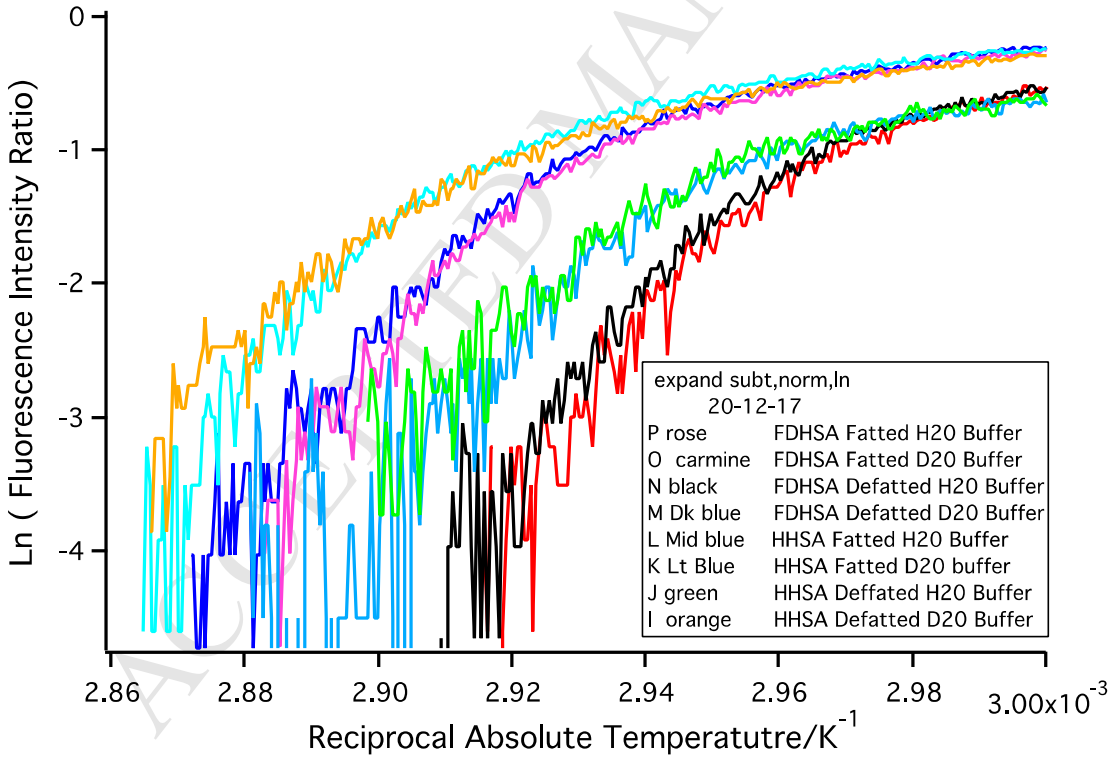


Figure 4 Van't Hoff melting curves for the eight recombinant proteins.

There are four groups and the almost co-incidence of fatted and defatted results – is clear. For the range -3 <ln(ratio) <-1.5 the slopes lead to ΔH with associated ΔS shown in **Table 3** . We quote the enthalpies of melting to ± 50kJ//mole for reasons of caution all are in the range quoted by Lang and Cole [11]. The ΔH for hydrogenous molecules are less negative than for the deuterated molecules by ca 50 to 100kJ/mole

and the effect of the deuterated buffer looks to be a stabilisation of ca. 50kJ/mole – except for I and J.

Table 3 van't Hoff Enthalpies and Entropies of Melting

		ΔH kJ/m	Tm/K	ΔS J/m/K
I - HHSA Defatted D_20 Buffer	orange	469	343	1370
J - HHSA Deffated H_20 Buffer	green	415	340	1220
K-HHSA Fatted D20 buffer	light blue	460	343	1340
L -HHSA Fatted H_20 Buffer	Mid blue	476	340	1400
M -FDHSA Defatted D_20 Buffer	dark blue	481	342	1410
N- FDHSA Defatted H20 Buffer	black	568	338	1680
O - FDHSA Fatted D_20 Buffer	carmine	517	342	1510
P- FDHSA Fatted H20 Buffer	rose	583	338	1730

Alternatively to the van't Hoff method of finding the slope we have used the whole melting function in upper part of Figure 3(a). This was fitted by equation 23 in [20] using MagicplotPro. Each sample I to P was fitted independently, and then the respective "defatted"/fatted pairs were averaged for the reasons explained above. The enthalpies are lower the above by about 100kJ/mole but the results are not far away from the published values of Picó [21] and Flora [22].

Table 4 (A) van't Hoff Enthalpies and Entropies of Melting

	ΔH [kJ/mol]	ΔS [J/K·mol]	T_m [°C]	T_m [°C][a]
HHSA/D_2O buffer (I/K)	204±3	593±10[b]	344±2	70.7±0.5
HHSA/H_2O buffer (J/L)	326±9	954±30[b]	342±4	66.9±0.1
FDHSA/D_2Obuffer (M/O)	239±3	699±10[b]	342±2	69.1±0.2
FDHSA/H_2O buffer (N/P)	326±6	965±20[b]	338±3	64.9±0.0
Defatted HSA (Flora/Trp)[d]	350	1050	60	
Defatted HSA (Picó/Trp)[c]	377		62.1	
Defatted HSA (Picó/DSC)[d]	376		63.2	

[a]T_m obtained from first derivative, [b] T_m calculated from $\Delta G = \Delta H - T_m \Delta S = 0$, [c][21], [d][22].

Table 4 shows that the values for ΔH are similar to those reported before by Picó and Flora for defatted HSA [(21,22] but fall on the lower end of a broad range of melting enthalpies reported by Lang & Cole for other recombinant HSA molecules [11], where, especially fatted HSA proteins show somewhat higher ΔH values. Provisionally, we accept the lower values produced by defining the background from the whole melting curves at the two wavelengths as a function of temperature as likely to identify the 1 and 0 positions around the melting transition given the wide spread of ΔH from the first method (subtracting a background defined by the lowest

point on the pre-calculated F350/330 ratio. Better statistics would achieve this. We give, however, a Table from the Lang & Cole paper for comparison.

Table 5 van't Hoff Enthalpies and Entropies of Melting for recombinant human serum albumins [11]

	T1/°C	T2/°C	Delta H/kJ/m	Delta SJ/m/K
Fatted HSA M human 86% mono, 2.2F	64.2	70.8	486±4	1440
Fatted HSA N human 97.7% mono, 4.4F	66.9	72.9	667±11	1962
Recombinant HSA E-1 97.3% 0.2	67.8	78.2	287±24	842
DefattedHSAI-1 human 99.5% 0.24	69.5	81.1	398±33	1162

Conclusions

The recombinant expression method of Belew et al [10] combined with a high salt medium has produced deuterated and protonated forms of human serum albumin of increased stability. These proteins show different melting points depending on the degree of deuteration in the molecule and in the solvent and this reproduces the qualitative effects seen by others for solvent and side chain deuteration in the melting points. The values of the melting enthalpies are in the ranges found by others [11,17,18,19] for a series of recombinant and native hydrogenous HSA and other proteins.

The literature describes effects on thermal denaturation, proteolytic cleavage and aggregation arising from intra-molecular deuteration and change of solvent to heavy water. That change combines a purely solvent effect (from the cohesive differences between H_2O and D_2O) with partial proton exchange in the molecular structure. The experiments on glutathione S-transferase (GST) by Brockwell et al [23,24] show the perdeuterated protein to be less stable to heat denaturation and cleavage than the proto form but the per-deuterated form associates into dimers and not the proto-form.

Van Well and colleagues' calorimetric studies [19] show that both hard and soft proteins are more stable against thermal denaturation in D_2O than H_2O and attribute this to a larger hydrophobic effect in D_2O on the nonpolar amino acids of the protein. Bhattacharya *et al.* [25] using circular dichroism conclude that heavy water causes a 20% decrease in the helical structure and, from a study of the solvent dynamics of the binding to coumarin 153 find a markedly retarded binding rate in D_2O compared to H_2O.

The clear H_2O/D_2O isotope effect on bovine serum albumin denaturation seen by Sabounji et al. [26] using far UV circular dichroism showed the protein unfolding pathways as a function of temperature. Below 50 C the protein conformations are similar in H_2O and D_2O but heavy water retards irreversible thermal denaturation above that temperature. By contrast, above 90 C, D_2O causes rapid denaturation of BSA but this is not observed in H_2O. Here we distinguish between deuterium solvent and intramolecular deuteration.

References

1. D. C. Carter, J. X. Ho Adv. Protein Chem. 1994, 45, 153-203
2. S. Curry, H. Mandelkow, P. Brick, N. Franks Nat. Struct. Mol. Biol. 1998, *5*, 827–835.
3. S. Curry Drug Metab. Pharmacokinet. 2009, 24 (4): 342–357.
4. A. W. Yee, M. Moulin, N. Breteau, M. Haertlein, E. P. Mitchell, J. B. Cooper, E. Boeri Erba, V. T. Forsyth, L. Fu, S Villette, S. Petoud, Angewante Chemie. Angew. Chem. Int. Ed. 2016, 55, 9292 –9296 DOI: 10.1002/anie.201602747.
5. S. J. Cooper, D. Brockwell, J. Raftery, D. Attwood J. Barber, J. R. Helliwell, 1998, Chem. Commun., 1063.
6. X. Liu, B. L. Hanson, P. Langan, R E. Viola, Acta Cryst. 2007, D63, 1000–1008, ISSN 0907-4449
7. M. Blakeley, S. S. Hasnain, S. V. Antonyuk, IUCr Journal 2015, 2, 464-474. DOI:10.1107/S2052252515011239.
8. J.W.White, D.Hess, J. Raynes, V. Laux, M. Haertlein, T. Forsyth, A. Jeyasingham. European Biophysics J. 2015, 44, 367-371S.
9. Institut Laue Langevin, Grenoble, Scientific Council Report 2015.
10. M. Belew, M. Y. Li, W. Zhang, K. Caldwell. Separation Science and Technology 2008, 43, 3134- 3153.
11. B. E. Lang, K. D. Cole Biotech Prog. 2015, 31, 62-69.
12. T. Arakawa, Y. Kita Biochim Biophys Acta. 2000;1479: 32–36.
13. J. C. Ang, M. J. Henderson, R. A. Campbell, J-M Lin, P. N. Yaron, A. Nelson, T. A. Faunce and J. W. White. Phys. Chem. Chem. Phys., 2014, DOI: 10.1039/C4CP00293H.
14. M.A. Evenson, H.F. Deutsch. Influence of Fatty Acids on the Isoelectric Point Properties of Human Serum Albumin. Clinica Chimica Acta 1978, 89, 341-354
15. Prometheus NT.48 www.nanotemper-technologies.com
16. A. Krezel, W. Bal, J. Inorganic Biochemistry 2004, 98, 161-166.
17. B. Vulevic, J. J. Correia, Biophysical J. 1997, 72, 1357-1375.
18. A. Hattori, H. L. Crespi, J.Katz Biochemistry, 1965, 4, 1213-1225.
19. Y. M. Efimova, S. Haemers, B. Wierczinski, W. Norde, A. A. van Well. Biopolymers 2006, 85, 262
20. M. R. Eftink, The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys. J.* 1994, 66**,** 482–501.
21. G. A. Picó, Thermodynamic features of the thermal unfolding of human serum albumin. *Int. J. Biol. Macromol.* 1997, 20**,** 63–73 (1997).
22. K. Flora, J. D. Brennan, G. A. Baker, M. A. Doody, F. V. Bright Unfolding of Acrylodan-Labeled Human Serum Albumin Probed by Steady-State and Time-Resolved Fluorescence Methods. *Biophys. J.* 1998, 75**,** 1084–1096.
23. D. Brokwell, L. Yu, S. Cooper, S. McLelland, A. Cooper, D. Attwood, S. J. Gaskell and J. Varner, Protein Science 2001, 10, 572-580.
24. D. K. Das, T. Mondal, U. Mondal and K. Battacharya, Chem. Phys. Chem. 2011, 12, 812-822.
25. M. Bhattacharya, N. Jain, and S. Mukhopadhyay. J. Phys. Chem. B 2011, 115, 4195–4205.
26. D. L. Price, L. Fu, F. J. Bermejo, F. Fernandez-Alonso, M-L. Saboungi, Chem. Phys. 2013, 424, 62-69.