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Letter

# Direct Detection of Bound Water in Hydrated Powders of Lysozyme by Differential Scanning Calorimetry

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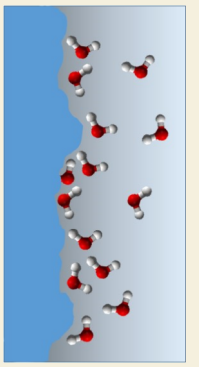
**ABSTRACT:** While exploring the behavior of lysozyme powders at different percentages of rehydration by differential scanning calorimetry, we noticed a small peak persistently on the left of the melting point of bulk water, which, when heating up the system, was always around −10 °C. The intensity of the transition was maximal at 160% rehydration and disappeared at higher values. By comparing the premelting peak properties in $H_2O$ and $D_2O$, we attributed it to freezable water bound on the protein surface. This is the first time that such an observation has been reported.



**KEYWORDS:** *bound and bulk water, hydrated protein powders, liquid-to-solid transition, protein stability, DSC measurements*

The importance of water for life as we know it can hardly be overstated. Water plays an essential role in all vital processes of living organisms and is the basis of life on our planet. It exists in different physical states—solid, liquid, and gas—and makes up 70% of the surface of Earth, plus 65−90% of the weight of all living organisms. At the molecular level, water plays an essential role in the properties of proteins, which, when not attached to membranes, are dissolved into aqueous solutions, whether they are in the cell or biological fluids. Water is also a crucial factor in protein unfolding, a process associated with an increasing number of pathologies, including Alzheimer's, Parkinson's, and other neurodegenerative diseases.[1]

A large portion of proteins have a well-defined three-dimensional structure, which determines their functions. When structured proteins are submitted to conditions far from their native environment, such as changes in temperature, pressure, chemical environment, or pH, they unfold and lose their functionality. Denaturation under heat conditions is a well-studied fact and widely used in processes of everyday usage, such as, for instance, pasteurization. In most cases, the temperature increase leads to aggregation and is irreversible. What is less known, mostly for the difficulty of observing transitions at temperatures below the freezing point of water, is that proteins can unfold also at cold temperatures,[2] generally reversibly. The driving mechanisms underlining the two processes are different: in both cases, water plays a dominant role. Heat denaturation is dominated by entropy as the temperature increases the motions of the protein, eventually leading to disruption of the intramolecular forces that keep the protein folded, exposing the hydrophobic core to the water. Depending on the circumstances, i.e., hydrophobicity, concentration, and environmental conditions, this promotes intermolecular interactions and aggregation. Enthalpy is more important at low temperature, where the hydrophobic forces weaken and the role of hydrogen bonding with the solvent becomes increasingly important, eventually determining loss of the native structure.[3]

In aqueous solutions, water encompasses proteins in different ways (Figure 1): water molecules all surround the protein and constitute what is called bulk water. Other water is motionally perturbed by the presence of a macromolecule but not bound to it and has a longer residence time[4] before mixing with the bulk water. This water is called the first hydration shell, or structured water. Finally, there is water tightly bound to the protein or trapped in it. This water has a much longer persistence time and was first observed in crystallographic structures of proteins.[5] It has in more recent times been extensively characterized by NMR and molecular dynamics studies.[6] Hydration water in the vicinity of a biomolecular "surface" exhibits dynamic properties different from those of the bulk.

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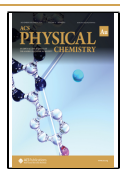





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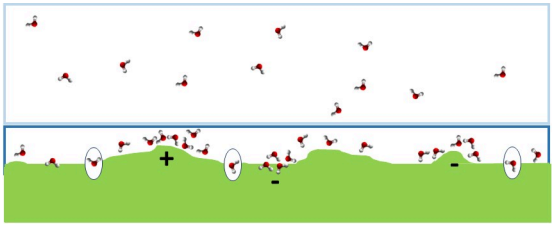

**Figure 1.** Schematics of three possible water populations. The protein surface is shown in green. The light blue box contains bulk water, and the dark blue box contains hydration water. The water molecules inside elliptic shapes are trapped molecules.

In the present study, we report an observation made using differential scanning calorimetry (DSC) to try to detect the cold denaturation of lysozyme. This is a small globular protein that is easily available and well characterized and, thus, especially suited for protein folding/unfolding studies. We used the protein in rehydrated powder form rather than in solution since powders provide a system widely adopted in investigations at temperatures below the water freezing point that permits the observation of several interesting phenomena: rehydrated powders can retain the native structure of proteins better than other forms (such as dried powders or crystal structures). Proteins in hydrated powders more closely mimic their native confined environment[7] compared to dried or denatured forms. Finally, understanding the behavior of proteins in rehydrated powders is crucial for drug development and biotechnological applications. It helps researchers optimize formulations, stability, and delivery mechanisms for protein-based drugs and biologics.[8]

We were studying lysozyme powders (20 mg) purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) (cat. no. L6876, lot #SLCK8560 at >95% purity by SDS-electrophoresis), used without further purification and rehydrated from 60 to 200 wt % $H_2O$, to check whether we could observe cold denaturation of the protein under any of these conditions (see the Supporting Information). DSC probes heat changes in relation with physical and chemical transitions, such as, for instance, the loss of tertiary structure or denaturation. While powerful, this method can suffer the problem of having to interpret the phenomenon which corresponds to the observed heat variation, which is usually done by assumptions rather than direct observations.

In our measurements, when going from low to high temperatures, we observed a main transition with peaks consistently around 0 °C that increased gradually as a function of increasing hydration (Figure 2a). These peaks had to correspond to the solid-to-liquid phase transition from ice to liquid water. Much to our surprise though, we also observed a second, much smaller peak around −10 °C (Figure 2b), which appeared starting at 60 wt % hydration, had a maximum at 160 wt %, and then decreased again. We first thought that this transition could correspond to the low temperature unfolding of the protein. Indeed, predictions of cold denaturation of RNAase[9] and staphylococcal nuclease[10] suggest values between −20 and −10 °C for this transition, close to our findings. However, the samples considered in the cited studies were in solution, and we reasoned that lysozyme is an extremely stable protein, having a heat denaturation midpoint of around or above 70 °C,[11] depending on the experimental conditions. As shown by Rüegg et al.,[12] the heat denaturation temperature decreases when hydration is increased. It has been

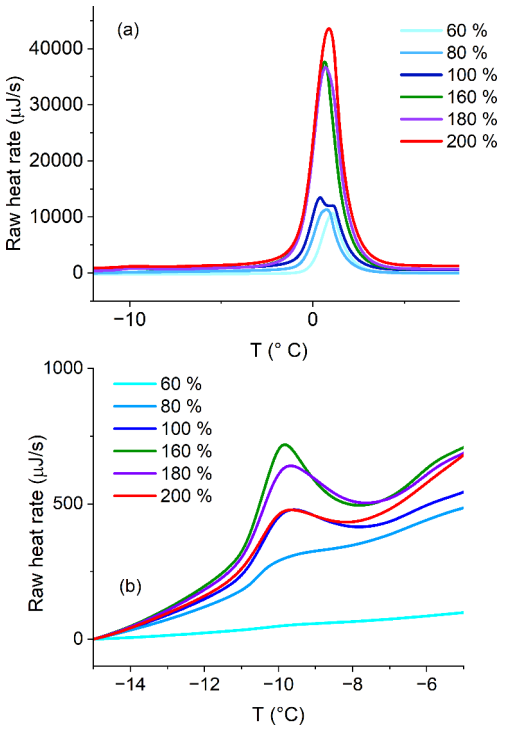


**Figure 2.** DSC measurements of the raw heat rate of lysozyme at different hydration levels (a) around the melting point of $H_2O$ and (b) around −10 °C (all curves were shifted to 0 at −15 °C for a better comparison).

demonstrated instead that cold denaturation detectable at observable temperatures is usually associated with only marginally stable proteins.[13] We thus considered different scenarios.

As an alternative, we formulated the hypothesis that what we were observing could instead be the solid-to-liquid phase transition of bound water as well, distinct from the transition of bulk water, which occurs, as we observed, around 0 °C. A possible way to explain the reduction of the −10 °C peak at high degrees of hydration could be the increasing exchange of bound water with bulk water due to the higher concentration of the latter that would result in the loss of detectability of the peak.

It is important to mention that, in these experiments, the scans recorded increasing the temperature were completely reproducible, with both the bulk water transition and the premelting peak appearing at exactly the same temperatures, i.e., around ∼ 0 and −10 °C, respectively (Figure 2). On the contrary, the cooling scans showed shifts in the transition temperatures: the bulk water transition occurred between −16 and −21.5 °C, which is well below the water freezing temperature (Figure S1). This is due to the well-known phenomenon of supercooling certainly, that is, the lowering of the melting point for kinetic effects, enhanced by the vibration-free environment needed for a DSC instrument and the use of small percentages of water in the powders. Accordingly, the premelting peak appeared between −19.25 and −23.75 °C. The spread of temperatures is explained by the fact that supercooling is a purely kinetic effect in which ice formation starts by the aggregation of water molecules at so-called nucleation centers whose formation is stochastic. We thus concentrated our analysis on the DSC thermograms going up, even though we kept recording both thermograms.


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To validate the hypothesis of a phase transition of bound water and be reassured that the premelting peak was associated with the properties of the water and not of the protein or impurities, we used the intrinsic difference between the properties of heavy water ($D_2O$) and $H_2O$. The freezing temperature of $D_2O$ is 3.8 °C, that is, ca. 4 °C higher than that of $H_2O$. Such a shift permits one to easily distinguish by DSC water from heavy water in a sample, although the isotope exchange is not completely anodyne, as mesoscopic structures and the elastic properties of biomolecular assemblies can differ in $H_2O$ from $D_2O$.[14]

We then fixed the percentage of hydration to 160 wt % (i.e., the degree of hydration with the maximal effect) for samples individually rehydrated with $H_2O$ or $D_2O$ (Figure 3 and Figure S2). The curve in heavy water was shifted by ca. 3.4 °C to higher temperatures. This difference is in excellent agreement with what was expected and clarified that we were certainly not observing cold denaturation because $D_2O$ is known to stabilize proteins as compared to $H_2O$ by tightening the hydrogen bonds.[15,16] The observed shift to higher temperatures in $D_2O$ thus indicated a clear relation between water and its phase transitions. The raw heat rates were converted to heat capacity $\Delta C_p$. The maxima of the two curves were similar in raw heat rate values, resulting in values of 46.200 $\mu$J/s in $H_2O$ and 45.800 $\mu$J/s in $D_2O$ (Table 1). The intensity of the premelting peak was of the order of 0.5% of that corresponding to the bulk water.

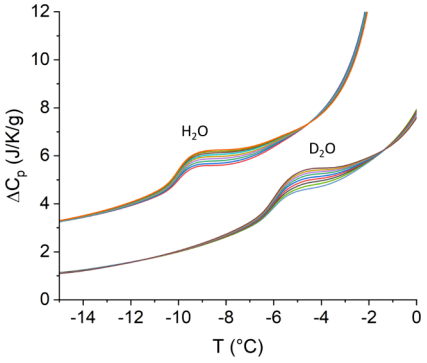


**Figure 3.** Heat capacity of lysozyme hydrated at 160 wt % in $H_2O$ (peaks centered around −9 °C) and in $D_2O$ (peaks centered around −5 °C), showing a displacement of ca. 4 °C. The consecutively measured peaks increase and shift to the right side.

Finally, we wondered whether and how much the detected processes were reversible. We found that the premelting peaks around −5 and −9 °C in $D_2O$ and $H_2O$, respectively, did not disappear when we did nine consecutive scans between −25 and 20 °C (Figure 4 and Figure S3). During the nine scans, the premelting transition shifted slightly to higher temperatures and the peak intensity increased, indicating some small rearrangements, albeit showing complete reversibility. On the contrary, when on the same sample we increased the temperature to 90 °C, an additional peak appeared at high temperature (Figures 4 and S3), around 70 °C (69 °C in $H_2O$, more difficult to assess quantitatively in $D_2O$, as it is shallower). This peak must reflect the heat denaturation of the protein, in agreement with literature values,[11] although the specific value depends significantly on the hydration state, the pH value, the presence of cosolutes, and other experimental conditions. When we went down to −25 °C, we could no longer observe the premelting peak at −9 °C, while the intensity of the transition at 70 °C was attenuated. This result proved the irreversibility of the high temperature unfolding, as it could be expected for protein aggregation caused by extreme conditions. It also showed that, once the protein is unfolded or aggregated, the premelting peak is lost. This reassures us further that the premelting peak could not come from impurities, since if it did, it would still be present after the loss of the native structure of the protein. A reasonable explanation for the disappearance of the peak after denaturation of the protein is that this transition causes a loss of the water trapped during protein folding in the protein cavities. Once lost, the water cannot be trapped again, and the process must be considered irreversible.

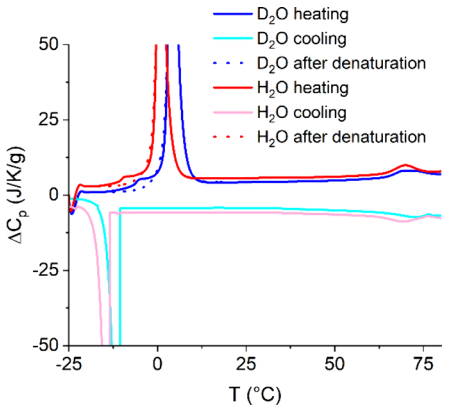


**Figure 4.** Heat capacity of lysozyme hydrated at 160 wt % in $H_2O$ and $D_2O$ heating up (red and blue profiles), cooling down from 90 °C (pink and cyan curves), and heating up again after hot denaturation. It is clear that, after reaching the high temperature unfolding above 70 °C, the premelting peaks disappear. The intensity of the transition at high temperature is also attenuated.

**Table 1. Thermodynamic Parameters Obtained for Lysozyme in $H_2O$ and $D_2O$ from DSC Data Analysis upon Heating According to the Measurement Cycle Described in the Supporting Information**

(a) Evolution between the First and Ninth Measurement Is Indicated for the Premelting Transition

| solvent | $T_{bound\ water}$ (°C) | $T_{heat\ denat.}$ (°C) | $\Delta H_{bound\ water}$ (J/g) | $\Delta C_p^{bound\ water}$ (J/K/g) | $\Delta H_{hot\ denat.}$ (J/g) | $\Delta C_p^{hot\ denat.}$ (J/K/g) |
|---|---|---|---|---|---|---|
| $H_2O$ | −9.6 → −9.4 | 69.8 | (1 → 2) ± 0.2 | (0.46 → 0.38) ± 0.04 | 21 ± 2 | 0.01 ± 0.8 |
| $D_2O$ | −5.4 → −5.3 | 69.1 | (1.8 → 1.9) ± 0.1 | (0.10 → 0.85) ± 0.03 | 14.4 ± 1.4 | 1.3 ± 0.6 |

(b) Evolution between the First and Ninth Measurement Is Indicated for the Bulk Water Transition

| solvent | $T_{bulk\ water}$ (°C) | $\Delta H_{bulk\ water}$ (J/g) | $\Delta C_p^{bulk\ water}$ (J/K/g) |
|---|---|---|---|
| $H_2O$ | 0.9 | 310 ± 5 | 2.0 ± 0.3 |
| $D_2O$ | 4.2 | 335 ± 5 | 1.5 ± 0.4 |



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The presence of bound water on the protein surface is not a new phenomenon: at low hydration, various scientists reported different water populations. In their X-ray diffraction studies on lysozyme crystals, Blake et al. described water bound to the protein in 1983.[17] The authors concluded that up to half of the water molecules were bound on the protein surface but not in a homogeneous way. The rest of the water was featureless and would correspond to what we named bulk water.

These experimental data were simulated by molecular dynamics (MD) with excellent agreement.[5] Poglitsch et al. studied picosecond relaxations by dielectric absorption measurements in low-hydrated lysozyme and found indications for bound water and N—H···O═C hydrogen bond variations with temperature, particularly below 0 °C.[18]

Celaschi and Mascarenhas[19] studied bound water at the surface of lysozyme at different hydration levels and with various techniques such as thermal-stimulated depolarization or pressure. They concluded that two types of "bound water" are found that can be distinguished by relaxation times and activation energies. However, the term "bound water" requires careful definition, as the hydration level must be sufficiently low not to cover all hydrophilic sites. Such populations cannot easily be observed with techniques other than NMR. At higher hydration, multilayers and liquid water appear. Kurzweil-Segev et al.[20] investigated confined water in hydrated lysozyme powders by DSC and found two peaks at 155 K (associated with the glass transition) and 186 K (whose origin is debated), but they did not discuss the temperature range up to 0 °C, which is the range we have covered.

We thus formulated the hypothesis that what we had observed in DSC thermograms is the solid-to-liquid transition of bound water. Alternative explanations are also possible, but they all need to involve the solvent, as we could see by substituting $H_2O$ with $D_2O$. This is an important observation because it has been long debated whether the different water populations are freezable and under which conditions. Kuntz and Kauzmann,[4] for instance, classified the bound water mainly as nonfreezable but without experimental bases.

An entirely different, but similar by analogy, study includes a description of the water observed by DSC in hydrogels at different hydrations.[21] The authors differentiated three water populations classified as bound water, which can be subdivided into nonfreezing or freezing bound water and free water. The authors observed a peak corresponding to the freezing bound water at low hydration levels, which increased and eventually disappeared at 250 wt %, resembling the behavior we observed. The exact transition temperature depended on the sample composition and the water content but was between −20 and −8 °C.

In conclusion, we can, under specific circumstances of hydration, observe bound water in a hydrated powder of lysozyme and its solid-to-liquid transition independently from the behavior of bulk water, as demonstrated by the properties of the DSC signal in $H_2O$ and $D_2O$. Our observation allowed us to extract the melting enthalpy and other thermodynamic parameters of the transition directly from the DSC profiles. This possibility could in the future prove helpful to understand further protein−solvent interactions. Also, our results repropose rehydrated powders as a unique tool that may allow evaluation of the hierarchical role of different water. Even more importantly, our data prove for the first time the existence of freezable water bound to a protein.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsphyschemau.4c00029.

Details about sample preparation, DSC experiments, and calculations of the thermodynamic parameters, as well as raw heat rates of samples (PDF)

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### Author Contributions

J.P.: conceptualization, data curation, funding acquisition, methodology, project administration, supervision, writing—original draft. K.K.: data acquisition. R.D.: data curation. E.S.: data acquisition and curation. A.P.: conceptualization, methodology. All authors contributed to the writing and approved the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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