Report on Ngai et al.: Change of Caged Dynamics at $T_g$ in hydrated proteins found after suppressing the methyl group rotation contribution"

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I comment here the initial version of the manuscript, before it was beautified by derivative enhancement. This method cannot be applied to data with the kind of noise achievable with neutron displacements. It was proposed by the second referee, which helped to hide the critical points of the analysis, as I will point out. The text is cut out from my referee report.

The manuscript presents a continuation of two very similar previous publications by the authors. Their latest paper, published in J. Phys. Chem. B, is cited fully in the Abstract but not in the reference list and is not mentioned in the text. The former paper, cited in the text as ref. 13, was published in an online journal, suggesting that the authors wanted to make sure that their controversial manuscript is published. The new manuscript reiterates the previous work of reinterpreting published elastic neutron scattering data of solvated proteins, the solvent is now restricted to hydration water. The conclusions are identical to those of the previous work, implying that the mean square displacements (MDS) of hydrated proteins plotted versus the temperature exhibits a kink at the glass temperature of the solvent $T_g$. Before I go to the details of whether this claim is new and convincing a few remarks are in order:

The authors belong to the elastic neutron scattering community, which intends to explain protein dynamics based on a single quantity, the MDS versus the temperature. This is exemplified in the title: Change of Caged Dynamics at $T_g$...

The restriction to the elastic domain and the MDS involves a drastic loss of dynamic information. The full dynamic information derivable from neutron scattering experiments is contained in the spectrum of inelastically scattered neutrons versus momentum exchange $Q$ (the dynamic structure factor) or equivalently the density correlation function (intermediate scattering function) in the time domain. The MDS approach ("displacementology") records only the small fraction of the spectrum near $\omega = 0$. This leaves the (in this case, incoherent) elastic scattering function versus momentum exchange to be analysed. For the MDS however, only the low $Q$ region of the scattering function, extrapolated to $Q = 0$, is evaluated. Thus the in general non-Gaussian nature of the scattering function is ignored. The next reduction concerns the temperature dependence of the MDS($T$). Here only the "onset of nonharmonic behaviour" at a particular onset temperature, the deviation of MDS($T$) from a straight line, matters. This is then "referred to in the literature as the protein dynamical transition".

It is obvious that with such a restricted view of protein dynamics errors and misconceptions are unavoidable. Some of these errors are now being corrected. The strategy is to sell the correction as a new discovery without citing previous work, where such a "discovery" was discussed years ago. This concept works remarkably well as long as some colleagues also play the game. The idea that some anomaly in the MDS of protein-water motions near $T_g$ exists is at least 25 years old. It was also discussed in their ref. 4, where the protein dynamical transition (PDT) was originally defined as a two-step feature. A recent paper on this topic entitled "the two-step scenario of the PDT" was initially not referenced by Ngai et al. in contrast to other less important papers in the same issue of JNCS (2011). There the onset approach of the MSD is strongly criticized. Now the most relevant paper of this article is referenced as number 35 part 2. This is a clear case of scientific misconduct.

The MSD approach of the PDT was initiated by a letter in Nature (1989) (their ref. 4) entitled "Dynamical Transition of Hydrated Myoglobin revealed by Inelastic Neutron Scattering". It was only a letter, but it displayed the temperature dependent spectra of hydrated myoglobin, combining data taken with two spectrometers together with a quantitative analysis of the
elastic scattering function and finally the resulting MSD(T). Two anharmonic onsets were recorded near T\textsubscript{g} and T\textsubscript{d}, where the second onset was assigned to the water-coupled and resolution dependent PDT. The first transition near T\textsubscript{g} (based on calorimetric and infrared data) was interpreted as a pre-transition due to fast H-bond fluctuations. Interestingly, the assignment was based on a high frequency spectral feature (called fast beta relaxation) and not just the MSD onset. It is remarkable that the elastic scattering community picked out of this letter only the MSD plot ignoring the inelastic information. This situation persists now since 1989. Consequently the present manuscript mentions only the elastic work with IN13 of ref. 4. The term "dynamical transition" refers to the glass transition of protein hydration water at T\textsubscript{g}, which is supported by respective calorimetric effects and changes of the thermal expansion coefficient. If recorded on the same timescale a discontinuity in the specific heat will occur also at T\textsubscript{d} (see ref. 33).

In 2005 it was shown by Doster/Settles (BBA) that the low temperature onset near T\textsubscript{g} was interfering with rotational transitions of side chains, mainly methyl groups. The unharmonic vibrational displacements of the protein-water hydrogen bonds are much smaller and are thus difficult to detect. The main goal of the present manuscript and the two previous papers by Ngai et al. is to identify the unharmonic MSD onset near T\textsubscript{g} without interference with methyl group transitions. I will investigate whether this goal is achieved. Their approach is purely qualitative, they look for small deviations of the MSD from harmonic behaviour near T\textsubscript{g}. The deviations are interpreted as reflecting a "general property of glass formers" without giving a detailed physical picture. This conclusion may apply to PMMA but it ignores the bio-literature.

Title
the authors to not provide any evidence for "caged dynamics" in the text, the concept remains vague, the conclusions on dynamics are based only on the MSD.

Abstract
Caged dynamics: in condensed matter not just in glass formers, the molecules are constrained by cages of their nearest neighbours. Dissolution of the cage in liquids specifies the alpha process and not beta-relaxation.

- it is misleading to state that the PDT was "first found" with Mössbauer spectroscopy in myoglobin crystals. The cited papers do not assign the observed unharmonic onset of local heme motions to a collective dynamical transition or a glass transition. Instead, the effect is discussed in terms of local motions between potential wells. With a fixed energy window method by varying the temperature any molecular process, which enters the energy window, will give rise to an nonharmonic onset at a particular temperature, for instance the onset due to methyl rotation. The relation between the Mössbauer effect of the heme iron and the hydrogen displacements observed with ENS is complicated. Not only the probe, the iron atom compared to an ensemble of protein hydrogen atoms, but also the Q-ranges are vastly different. It is questionable, whether one can derive a zero Q extrapolated MSD from a single Q\textsuperscript{2} = 50 Å\textsuperscript{-1} (as compared to 0.05 with NS) without intermediate data. Instead of a precise definition, the authors present a vague concept of the PDT. This is also true for the MSD itself. It is not clear what <u\textsuperscript{2}> really means, which is not defined here. I would not be surprised if the MSDs presented in the figures were evaluated with different meanings of <u\textsuperscript{2}> (ref. 24). It is striking that the only and basic physical quantity used in this text is not properly defined.
To conclude:
on the positive side:
1) the authors revise their previous view and now propose a more realistic concept of the PDT as a two-step process. This contrasts positively with numerous papers of the elastic scattering community. "Nevertheless, not all is lost" (p22), by the reinterpretation, the authors are trying to preserve the validity of their published data.

2) The close relation between the PDT and the glass transition is appreciated. $T_g$ and $T_d$ are correctly distinguished, they are interpreted as MSD onset-temperatures, which is questionable.

on the negative side:

1) The authors fall into the same trap as formerly Doster et al. 20 years ago (ref. 4): The enhancement of MSD near $T_g$ is a subtle effect, which is not easily spotted by the crude methods employed here. It is not sufficient to avoid the contribution of methyl groups. A physical concept is missing, the notion of a general effect near $T_g$ is not convincing. The MSD enhancement is always related to a molecular process. The onset near $T_g$ depends of course on the experimental resolution, as in the case of calorimetry. The apparent independence simply points to ultra-fast motions, which excludes the GJ beta relaxation.

2) The data analysis presented here is crude and outdated. A new approach, which avoids the arbitrary assignment of onset temperatures, was proposed in ref.0 in 2011. It is thus not sufficient to dig out old data by performing a "break dance". Knowing that, is probably the main reason, why the authors do not cite ref. 0.

In fact, the authors provide little convincing evidence in support of the onset of MSD near $T_g$. Alternative and more logical explanations of the MSD onsets can be easily given. Not even the claim of suppressing methyl group contributions is always correct. By contrast, other authors, which did provide evidence of such effects, are not cited as discussed above.

A more detailed discussion of this work was given in Doster et al. JCP (2013) 139 145 105,

In fig. 12 it is shown, that displacements strongly depend on the respective Q-range and of course resolution. It is suggested to use the complete elastic scattering function, not just their low Q limit.

Figure 12 Doster et al. JCP (2013) : Apparent 1-D mean square displacements of 100 % glycerol and lysozyme in glycerol 50:50 at high Q: blue squares: glycerol$^1$, red circles: lysozyme/glycerol$^{19}$, and at low Q: open squares: partially deuterated glycerol$^5$, open circles: lysozyme/glycerol 50:50.