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Response to the "Comments on 'Statistical thermodynamics of casein aggregation: Effects of salts and water' [Biophys Chem. 247 (2019) 34 − 42] "

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While Horne's Comments [1] are written with expertise in dairy protein solutions, they nevertheless contain numerous fundamental errors, not only of our theory but also of our scope. The aims of our Response, therefore, are:

A. to point out and clear up Horne's confusion with our theoretical foundation, the basis of his criticism;
B. to stress why statistical thermodynamics is necessary to link macroscopic phenomena and molecular behaviour and highlight its advantages;
C. to recount the strengths and limitations of our approach;
D. to clarify that Horne discusses a different scale from our work – and to show that linking the two will yield a full understanding of the aggregation phenomena.

The overarching approach is our original aim: to make analysis of casein systems feasible via statistical thermodynamics.

Theoretical foundations

In our analysis, we use Kirkwood-Buff Integrals (KBI; Eq. (5)): the established [2–4] universal tool to describe molecules and their systems. KBIs can

i. be directly interpreted based on the definition (Eq. (5) of [5]);

ii. provide information on net (overall) distribution of a species around another;

iii. provide a direct and quantitative link between intermolecular interactions and aggregation;

iv. be applied regardless of the strength / specificity of interactions.

Basing on these essentials, we can examine whether our theory is "fatally flawed" [1] as Horne claims. .

Point A: Theoretical foundations

The origin of the criticisms presented in Horne's Comments is overlooking the theoretical foundations of the Fluctuation Adsorption-Solvation Theory (FAST) [5]. In paragraph 4, he states that we treat proteins as "structureless, featureless points or spheres" [1]. This is a misunderstanding. As has been well-established in the literature, KBIs are defined universally for any molecule [2,6–8], globular molecules equally to intrinsically disordered ones.

Horne declares (paragraph 9) our principal failing to be assuming non-specific interactions of calcium and casein. He states that the interactions are highly specific [1]. However, first, ions interact with proteins in specific and non-specific manner [9]. Second, KBIs, because of the generality (Eq. (5)), deal with specific *and* non-specific interactions. There is no limitation coming from strength or specificity [8,10]. Thus, our interpretation of interactions is valid in each case.

Moreover, FAST "approach […] can be extended to the colloidal stability" [5] (p 39), namely to aggregation (and consequently flocculation [11]) driven by micelle-micelle interaction, what already has been shown in literature [12,13]. Therefore, Horne's allegation "that caseinate is already in an aggregated form before calcium addition […] somehow has escaped Harton and

Shimizu's attention" [1] (paragraph 6) derives from his erroneous comprehension of the theory, and is groundless.

Point B: Necessity of KBIs

Horne states we "offered no experimental evidence in support of this assertion" and "no evidence or citation to binding studies" when discussing role of salts in aggregation (paragraphs 2, 5) [1]. Our assertions [5] come directly from values of KBIs and are derived from the very definition, Eq. (5) [5,8]. Hence, his criticism is, again, a basic misunderstanding of our scope and method.

Horne rightly brought up electrostatic interactions as playing an important role in elucidating the effect of ions on aggregation (paragraphs 7, 8) [1]. But there are diverse interactions at work: hydrogen bonding, ion bonding, hydrophobic interactions, van der Waals forces [14–16]; Horne implied that calcium is the driver (paragraph 9) [1]. The crucial aspect is that all these interactions take place in water. Water molecules surround proteins and ions – ion-protein interactions are mediated by them [5]. Therefore, the key to understanding aggregation is understanding how water mediates intermolecular interactions in the system.

KBIs are a quantitative link between net interactions and aggregation from the principles of statistical thermodynamics [2,8]. "Quantifying both [protein-water and protein-cosolvent] interactions independently from the perspective of solvation" [5], we answered the main question, what is the role of water in casein aggregation: ion-induced hydration changes are negligible. Strength of contributions – not only of detailed by Horne calcium, but also water, and NaCl, NaSCN, $NaClO_4$, TFA – came out of our analysis quantitatively.

Point C: Scope, approximations, and limitations

We have established and underscored in our original message that scrupulous KBI calculation and interpretation requires different data than have been reported in the literature of dairy sciences [5]. Following our goal of streamlining analysis of casein systems, KBIs can be determined based solely on attainable experimental data of simple solvent mixtures. Model approximation was applied to obtain tractable and realistic first step. Consequently, we adapted "another casein, α_{s2}" as a functional example. Of course, we are aware that "[t]he thermodynamic models we have employed in this paper, such as the isodesmic model, suffer from limited applicability" [5]. Furthermore, we decided to analyse the results of Dickinson et al. approximately, for low Ca^{2+} concentrations, rather than to interpret irrelevant data. We continue in our wish of "[e]xtending our theory to concentrated micelle solutions under increased ionic strength" with "a thermodynamic model for polydisperse micelles" [5].

In paragraphs 5 and 6 Horne censures us for ignoring use of $NaCl/CaCl_2$ buffer and resulting changes in ionic strength, casein micelles stability and structure, and impact of temperature [1]. All these criticisms are amiss. In Section 3.1 (p 39) we have clearly stated the presence of $NaCl/CaCl_2$ buffer[5]; we acknowledged that aggregation is for fully-rennetted caseins and thus the results are valid for the initial stage of the process [5]; anomalies with respect to temperature are beyond the scope of our analysis, nonetheless also are remarked [5].

What is more, none of these impede our interpretation. Concentration of calcium is virtually constant, while the change in ionic strength comes from the variable, NaCl. As stated by Dalgleish, calcium is crucial for the aggregation to take place [17]. Hence, its presence cannot be omitted [16] and so, all experiments require Ca^{2+} in the solution. Our analysis was under

constant temperature (p 35) [5]. Moreover, Horne confuses the matter with his allegations on caseinates: in Section 3.2, on colloidal stability of caseinates, we specify that we examine caseinate-caseinate interactions (p 39) [5]; not casein monomer to caseinate process. We are therefore in unison, "caseinates are already in an aggregated form before calcium addition" [1].

Horne justly pointed out that there is abundance of studies of casein aggregate architecture, however, the validity of the calculated KBIs does not depend on this aspect of molecular detail.

Point D: From KBIs to microscopic understanding

Horne states that we are not addressing our "contradictory results" (paragraphs 6-8). However, *kinetics* and *thermodynamics* of aggregation, as stated in Section 2.3, are different [8,13,18,19]. Different phenomena require different theoretical approaches, and yield different results [8,13,18,19].

In fact, clarifying Horne's criticisms shows further advantages of statistical thermodynamics. Horne states that presence of "naturally bound" Ca^{2+} in casein micelles impairs our analysis. This is not true. KBIs already incorporate the residual Ca^{2+} into the "*p*" species, and the influence of NaCl concentration is on entire micelle, casein + residual Ca^{2+}. It must have escaped Horne's attention.

By extension of our theory, we can overcome the limitation of the traditional approaches that "NaCl influences both the range of electrostatic repulsion as an ionic strength effect and the magnitude through altering the calcium binding, making a more quantitative analysis difficult" [1].

Our conclusion regarding the interaction of Ca^{2+} with the transition state is consistent with Dagleish's proposed acceleration based on surface charge neutralization [5,16]. Hence, a microscopic interpretation of KBIs successfully completes the conjecture [5]. Furthermore, going beyond the scope our original paper, here we estimate the contribution of Ca^{2+} on the effect of Na^{+} in aggregation kinetics. Neglecting small contributions from $\Delta G_{p1}^{\ddagger}$[5], the dependence of casein aggregation (kinetics) on NaCl concentration (m_2) in the presence of a constant $CaCl_2$ (m_3) is expressed as:

$$-\frac{1}{RT}\left(\frac{\partial \Delta\mu_p^{\ddagger}}{\partial m_2}\right)_{m_3} \simeq \frac{c_2}{m_2}\Delta G_{p2}^{\ddagger} + c_3 \Delta G_{p3}^{\ddagger}\left(\frac{\partial \ln a_3}{\partial m_2}\right)_{m_3} \quad (1)$$

where species "3" refers to $CaCl_2$ and used $\left(\frac{\partial \ln a_2}{\partial m_2}\right)_{m_3} \simeq \frac{1}{m_2}$ for dilute salts. Note that have used the molality concentration for the ease of handling the activity data [20]. Let us roughly estimate the contribution from $CaCl_2$ (the final term). Under the condition $c_3 = 5\ \mathrm{mM} = 5 \times 10^{-3}\ \mathrm{mol\ dm^{-3}}$ [16] and using $\Delta G_{p2}^{\ddagger} = 4.84 \times 10^{1}\ \mathrm{dm^3 mol^{-1}}$[5], we obtain $c_3 \Delta G_{p3}^{\ddagger} = 0.24$. Using Eq. (19) of Robinson and Bowler [20], we can estimate $\left(\frac{\partial \ln a_3}{\partial m_2}\right)_{m_3} = \left(\frac{\partial \ln \gamma_3}{\partial m_2}\right)_{m_3} = 0.15\ \mathrm{kg\ mol^{-1}}$. Thus, the second term of Eq. (1) is $0.036\ \mathrm{kg\ mol^{-1}}$, which is two orders of magnitude smaller than the first term, $-2.15\ \mathrm{kg\ mol^{-1}}$, calculated from $\Delta G_{p2}^{\ddagger} = -2.15\ \mathrm{dm^3\ mol^{-1}}$ multiplied by $\frac{c_2}{m_2} \simeq 1\ \mathrm{kg\ dm^{-3}}$. Note, that our estimation on the calcium effect refers to the upper limit; according to Horne [1], the dissociation of Ca^{2+} along with the increase of Na^{+} would decrease the Ca^{2+} contribution. This, yet again, validates our preliminary calculation [5].

Furthermore, calcium binding studies of α_{s1} and β-caseins provided by Horne [21,22] showcase that traditional approaches of mathematical intricacy have difficulties in turning data into interpretation. It comes from inherent problems in managing a number of model

assumptions that were used to construct binding models, followed by cumbersome fitting procedures [21,22]. FAST, in contrast, provides a simple, direct and approximation-free link between the proportion of bound salt, ν, and KBI, as $\nu = c_2(G_{p2} - G_{21}) \simeq c_2 G_{p2}$ [10,23], showing how the chemical potential of unassociated protein changes with salt concentration. However, analyzing how ν depends on c_2 [21,22] by principle cannot yield protein-protein interaction (virial coefficients or multiple-body KBIs [12]) that govern precipitation [24]. Instead, it yields higher order correlations involving salts or water, as shown by the previous work by one of us [12,25]; comparing the strength of binding of first and subsequent Ca^{2+} [21,22] merely leads to cosolvent-cosolvent correlation in the presence of a protein. Hence, despite their in-depth analysis, protein-protein G_{pp} – a minimum requirement towards the elucidation of precipitation – cannot be obtained by approach presented in literature provided by Horne.

Thus, the two additional examples provided by Horne provide further evidence that KBIs are vital in analysis, being a quantitative tool to link experiments to molecular mechanisms.

Conclusion

Comments by Horne present number of misunderstandings which herewith have been clarified. Our goal was to create a pragmatic model for tractable approximations to interpret molecular behavior basing on empirical data. We have achieved this goal by principles of statistical thermodynamics. KBIs relate total specific and non-specific interactions, and analyse the system quantitatively. Consequently, we compared contributions of salt and water in the process of aggregation of casein – we found that role of water molecules, alteration of hydration, is negligible, and we enumerated the contribution of particular salts. We indeed used *different* type of experimental data; but thus, we provided useful estimates on interaction

strengths. These estimates introduce more lucidity into casein aggregation processes, complementing pre-KBI data, and prepares the way of a full understanding that is to come.

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