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A balanced view of casein interactions

David S. Horne *

Wisconsin Center for Dairy Research, University of Wisconsin—Madison, 1605 Linden Drive, Madison, WI 53706-1565, USA

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ABSTRACT

In this short review of hydrophobicity in relation to the caseins and their properties, it is concluded that the scales are weighted heavily in favour of hydrophobic interactions contributing the attractive component in casein self-assembly and casein micelle formation. Multiple clusters of hydrophobic residues are identified as potential reaction sites in the hydrophobic tails and trains of the casein proteins. Multiple examples of the involvement of hydrophobic interactions are listed. The concentration of electrostatic charge in the phosphoserine clusters of the proteins amplifies the range of electrostatic repulsion. The cooperative, concerted nature of the phenomenon takes hydrophobic interaction to a similar operating range to provide the perfect foil to the repulsion.

1. Introduction

Casein micelles are the aggregates of the casein proteins with mineral calcium phosphate found in the liquid milks of all mammalian species. How these micelles are formed, how they respond to the various treatments applied in dairy product manufacture are controlled by the various interactions between the protein chains. Recent reviews by Holt and colleagues [1–3] have questioned the involvement of hydrophobic interactions in the association of the caseins and in casein micelle formation. Equally, others [4•,5,6•,7] have continued to adopt the historic line that hydrophobic interactions are of major importance in casein aggregation and micelle assembly.

The arguments advanced by Holt and colleagues [1–3] for downgrading hydrophobicity are weak and open to challenge. In their discussion of the interactions prevailing within the casein micelle, Holt and colleagues appear now to fully subscribe to the polymerizing dual-binding model of Horne [8]. They retain the linkages between the phosphate centres of the casein and the calcium phosphate nanocrystals, although they continue to view this interaction as an adsorption process onto the nanocrystal rather than the active initiator/terminator roles proposed for the phosphoserine clusters by Horne et al. [9]. Rather than follow the historical picture of segregative hydrophobic trains and tails first recognized by Swaisgood [10], Holt et al. [1] re-labelled these regions as proline/glutamine rich, or "sticky P,Q-rich regions", which become "polar tracts" in Holt [3], this despite the dominance of *non*-polar, hydrophobic amino acid residues in these peptides. Holt [3] claims justification for use of the phrase "polar tract" is given in Holt et al. [1], but a word search of this paper fails to find mention of it.

The attempts by Thorn et al. [2] to identify the source of the "stickiness" of the P, Q-rich regions are confusing and lack clarity. Indeed, they present a veritable kaleidoscope of smoke and mirrors, a hotch-potch of ellipsis and omissions. Their suggestion of "main-chain-to-main-chain interactions of low sequence specificity" is claimed to be based on comparison with the behaviour of "similar unfolded proteins" but the example they provide is of WW-domains with a triple β-sheet configuration interacting in a highly specific manner with a particular polyproline peptide of defined sequence [11]. Neither the WW-domain nor the proline peptide sequences are to be found in any bovine casein.

Thorn et al. [2] offer the argument that hydrophobic interactions and the formation of a main-chain-to-main-chain H-bonded network have similar thermodynamic signatures, citing Cooper [12,13] in support. First this betrays ignorance of the modern view of hydrophobic interactions (see Section 4), and second Cooper emphasizes that account must be taken of all possible H-bonding in the system, including that of solvent water. Cooper [13] also states "changes in heat capacity quantitatively similar to those seen in biomolecular processes are an inevitable consequence of any system involving co-operative transitions of a multiplicity of the nature of the interaction". Ambiguity simply means we cannot argue for one possible interaction over another.

Holt et al. [1], repeated in Thorn et al. [2], suggest that the interactions between P,Q-rich regions be termed "entropic interactions". This cannot be accepted, as this terminology is already applied in colloid and polymer science (eg Klein [14,15] where it refers to interactions involving changes in configurational entropy of adsorbed or interacting polymers. Confusion could therefore arise but more generally, it is the free energy, ΔG, including both enthalpic and entropic effects that will determine the outcome of any interaction.

Rather than accept this situation and noting significant omissions in the arguments of the Holt/Thorn papers, notably in lack of consideration

* 2 Boghall Farm Steadings, Bathgate, West Lothian, EH48 1LQ, Scotland, UK.
E-mail address: brendavi@aol.com.

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of protein charge, I propose to review casein interactions in biophysical terms. I start by considering hydrophobicity, a much-maligned concept in the Holt/Thorn reviews, in Section 2. Section 3 looks more closely at the casein sequences, the distribution of charged, polar and non-polar residues, and how these distributions define the interactions of the caseins. Section 4 then considers *all* possible forms of non-covalent bonding between interacting proteins, convincingly refuting the criticisms of Holt et al. [1] and Thorn et al. [2] regarding the involvement of hydrophobic interactions between caseins whether in the casein micelle or in aggregates of individual proteins.

2. Hydrophobicity

Arguably the most important portion of an amino acid is its side-chain. The side-chains differentiate the amino acids from one another. It is the chemistry of the side-chains and the sequence of amino acids that govern the structure, function and purpose of a protein. In turn, the most important attributes of a side-chain are its electric charge and its hydrophobicity. Ironically for Thorn et al. [2], who are dismissive of the importance of hydrophobicity in the caseins, which they wish to be viewed as intrinsically disordered proteins, the distinguishing features of this class of proteins are a high average net charge and a low average hydrophobicity [16].

Because of its recognized, obvious importance, much effort has gone into attempting to provide a quantitative, numerical scale of hydrophobicity. The difficulty here is in deciding on the ruler. One of the first such scales [17] was based on the free energy of transfer of amino acid side chains from ethanol to water, with glycine, the amino acid with no side chain being taken as the zero point of the scale. It was this scale that Bigelow [18] used in his calculation of the average hydrophobicity of casein, an honest calculation based on compositional data available at that time. This placed α-casein in the top 10% of the 150 proteins in his list. This average hydrophobicity data is discussed in greater detail in Section 3.

The Tanford [17] scale is open to criticism from several aspects. Tanford noted that transfer free energies were not available for five amino acids, cysteine, cystine, histidine and charged glutamate and aspartate residues, but his table has no listings for glutamine, asparagines or serine. Moreover, the positively charged polar amino acids, lysine and arginine, are significantly more hydrophobic than glycine in this method of computing amino acid hydrophobicity.

Further attempts at constructing a scale of hydrophobicity followed and by 1987 Cornette et al. [19] were able to compare the 38 previously published scales for their ability to identify the characteristic period of α-helices in protein sequences. Attempts continued so that by the beginning of the millennium over 100 scales of hydrophobicity/hydrophilicity had been constructed [20•]. Partitioning between two immiscible liquid phases is the most common method of measuring hydrophobicity though non-liquid phases such as vapours or micellar phases have also been employed. With the availability of larger libraries of protein structure, methods have been developed to estimate amino acid hydrophobicity based on the degree of exposure of amino acid to solvent. Chromatographic methods using peptides or derivatized amino acids as solutes relate amino acid hydrophobicity to the retention of the peptide. Several scales have been created from measurement of a particular physical property of the amino acid e.g. surface tension [21], and others using site-directed mutagenesis [20•]. Theoretical molecular dynamics has also been employed more recently to develop an understanding of hydrophobicity through solvation studies [22••] with theory here bearing on the emerging theoretical basis of hydrophobic interactions (Section 4).

The wide range of techniques applied to the determination of amino acid hydrophobicity attest not only to the importance placed on knowledge of this parameter but also to the pervasiveness of its influence. Because different scales frequently rank the amino acid hydrophobicity values differently, histograms of these rankings, indicating the frequency of occurrence in a particular position on the scale, can prove enlightening [20•]. From these histograms (examples in Fig. 1), we can calculate a number-average mean ranking for each amino acid, and thereby derive, as it were, a global ranking for the hydrophobicity of the naturally occurring amino acids (Table 1).

The listing is as might generally be expected; the non-polar amino acids with the most hydrophobic side-chains appear in the top-most regions of the table and the charged amino acids at the bottom. More subtle expectations are also satisfied. Alanine has one more CH_2-group than glycine and appears higher in this table. Ile and Val both possess β-branched side-chains but Ile has an additional CH_2-group, raising the expectation that Ile would be more hydrophobic, as listed. Similarly side-chains with functional groups would be expected to be more hydrophobic if they had an additional CH_2, and as expected the tables generally place Thr more hydrophobic than Ser and Glu more hydrophobic that Asp, as reflected by their mean ranking values (Table 1).

Vast though the literature on hydrophobicity scales may be, it is also apparent that a consensus on absolute values or rankings is some way off. It may be that this is not a realistic goal in view of the latest notions on hydrophobic interactions and of the number of parameters involved but the fact remains that hydrophobic interactions remain centre stage in many studies of protein assembly, aggregation or folding.

3. Caseins

3.1. Hydrophobicity and electric charge

Holt et al. [1] have asserted that "the reputation of the caseins as hydrophobic proteins is undeserved". We can find no support for this assertion in the literature. Fox and Broadkorb [23], the supporting citation of Holt et al. [1], simply say that β-casein is the most hydrophobic of the individual (bovine) caseins with no further comment on the standing of the caseins relative to other proteins. What, perhaps, Holt et al. [1] were trying to say is that we should not think of the caseins as hydrophobic proteins, for there is some evidence supporting that position.

The caseins are not bereft of the recognized hydrophobic residues, I, L, F, W, M, Y and V. Examination of their amino acid compositions reveals that 28% of κ-casein, 30% of α_{S2}-casein, 32% of α_{S1}-casein and 34% β-casein (all bovine) residues are hydrophobic, i.e. approximately 1 in 3. Utilizing the values tabulated by Bigelow [18] for the hydrophobicities of individual amino acids, Swaisgood [10] calculated the average

Table 1
Global mean ranking computed from frequency histogram data of Biswas et al. [20•].

Position	Amino acid	Mean ranking
1	Trp	3.81
2	Phe	4.00
3	Ile	4.21
4	Leu	4.62
5	Tyr	6.7
6	Val	7.06
7	Met	7.24
8	Cys	9.29
9	Pro	9.65
10	Thr	10.1
11	Ala	11.45
12	His	11.97
13	Gly	13.09
14	Arg	13.36
15	Ser	13.47
16	Lys	13.69
17	Gln	13.82
18	Glu	13.83
19	Asp	14.97
20	Asn	15.19
21	SerP	Not Available