1
2 *Original Research Article*
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Theoretical Insight into Preferential Interaction Issues and Solution Structure, and Contentious Apparent Hydrated Molar Volume of Cosolute.

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ABSTRACT

Background: There seems to be a mathematical or a conceptual error in an equation whose substitution into other equations for the determination of an apparent hydrated molar volume (V_1) of a cosolute leads to an incorrect answer.

Objectives: The objectives are 1) To show theoretically that the preferential interaction parameter (PIP) is an extensive thermodynamic quantity, 2) rederive new equations and reexamine various equations related to solution structure, 3) apply derived equation for the determination of V_1, and 4) determine m-values and cognate preferential interaction parameter (PIP).

Methods: The research is mainly theoretical and partly experimental. Bernfeld method of enzyme assay was adopted for the generation of data.

Result and discussion: The investigation showed that equation linking chemical potential of osmolyte to solution structure is dimensionally invalid; PIP was seen as a thermodynamically extensive quantity. Equations for the graphical determination of V_1 of the osmolyte were determined. With ethanol alone, there were $-$ m-value and $+$ PIP; with aspirin alone, there were $+$ m-value and $-$ PIP. There was a change in sign in m-value with

sucrose and ethanol/aspirin mixture, and a change in sign in PIP when the latter is taken as function of [ethanol]/[aspirin] and [sucrose](C_3).

Conclusion: A solution structure is as usual determined by either a relative excess or a deficit of the solution component either in the bulk or around the macromolecular surface domain; the PIP remains thermodynamically an extensive quantity. To be valid there is a need to introduce a reference standard molar concentration or activity to some equations in literature. The slope $\partial\left(\frac{\gamma_3-1}{\gamma_3}\right)/\partial C_3$ from one of the equations seems to give a valid value for V_1 (V_1 is «1; γ_3 is activity coefficient). A known destabiliser may behave as a stabiliser being excluded. Like ethanol, aspirin as cosolute is destabilising and opposed by sucrose.

Keywords: *Keywords: Porcine pancreatic alpha amylase; preferential interaction parameter; apparent hydrated molar volume; m – value; Kirkwood-Buff integrals; ethanol; aspirin; sucrose.*

1. INTRODUCTION

For many years according to Schurr et al [1], scholars have presented a theoretical discourse on the concept of cosolute (or cosolvent otherwise known as osmolytes that are the organic and inorganic compounds) preferential interaction with macromolecules. There are several equations defined by the use of different symbols but all addressing the same issues. The issues are mainly solution structure, the change in such structure whenever an osmolyte or a macromolecule is introduced into any of such solution; the effect of the osmolytes on the macromolecular three dimensional (3-D) structure is often investigated using various biophysical instrument amenable to mainly biophysical studies [2]. There is also an attempt to link the interaction parameters to Kirkwood-Bulk integrals and *m*-value (this is the slope of the plot of free energy of folding to unfolding transition versus cosolvent concentration) [3-7]. The catalytic activities of the enzymes are also studied in the presence

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28 and absence of the osmolytes with the hope of understanding or establishing the effect of
29 thermodynamic temperature increase in particular may be on the function of the enzyme [2,
30 8]. There were theoretical studies in the past [3, 9] all geared towards gaining theoretical
31 insight into the solution structure and thermodynamic properties. It seems that there are far
32 more biophysical studies than purely biochemical studies at the experimental front. Yet it is a
33 greater theoretical insight that can facilitate the interpretation of results. Hence this research
34 is mainly theoretical with minor experimentation for the generation of data for the evaluation
35 of the derived equations.

36 Scholars have explained the mechanism of preferential interaction of osmolytes with
37 biomolecules often in the usual consistent way [3, 7, 10]. While preferential binding
38 (otherwise called solvation by binding) leads to unfolding that accompanies displacement of
39 water of hydration and perhaps water of preferential hydration, preferential hydration leads to
40 the folding of unfolded protein. The folding of the unfolded protein results from the
41 preferential exclusion of the osmolyte from the surface (the peptide back born) of the protein.
42 Recently, a different mechanism as opposed to preferential hydration has been advanced for
43 the (re)folding of biomolecules [11]. The Lifshitz's dispersion forces play a strong role in
44 solute-induced stabilisation/destabilisation of globular proteins [11]. The positive and/or
45 negative electrodynamic pressure (perhaps due to such forces) generated by the solute-
46 protein interaction across the water medium seems to be the fundamental mechanism by
47 which solutes affect protein stability [11]. There is also the concept of translational entropy
48 (TE) [12] regarded as the driving force that opposes conformational entropy connected to
49 unfolding thereby forcing (re)folding. Hydrophobic effect is also known to promote folding
50 [11, 13].

51 The issue remains effects of hydration and solvation or osmolation. But there are
52 models used to separate the effect of hydration from those of solvation of proteins. Those
53 models according to Rösgen *et al* [3-7] are the exchange model, osmotic stress model, local
54 domain model, and constant solvation model. There is an attempt to bypass model-

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dependent assumptions while targeting Kirkwood-Buff (KB) – based protein solvation model to describe protein stability [3]. However, there seems to be an error, typographical or conceptual in nature. Most of the models are at the far end of biophysics with cognate biophysical methods. The hi-tech instruments for achieving the intended measurements are those for circular dichroism spectroscopy, infrared spectroscopy, differential scanning calorimetry, Fourier transform infrared spectroscopy *etc* [2]**.** An example of biochemical method is the assay of any enzyme whose velocity of action can be monitored using spectrophotometer of any kind that may be suitable. Adequate understanding of the issues regarding preferential interaction parameters, protein folding, and unfolding or misfolding are important to biological scientist, biochemist, pharmacist *etc*. This is so because of the effects that may be (in) compatible to health. To this end, there is a need to achieve greater theoretical insight regarding molecular interaction through far reaching or robust analysis of the issues involved. There is a need also to shift from so much emphasis on biophysical approaches to biochemical methods.

The objectives of this research are: 1) To show theoretically that the preferential interaction parameter (PIP) is an extensive thermodynamic quantity, 2) rederive new equations and reexamine various mathematical equations related to solution structure, 3) apply derived equation in the determination of apparent hydrated molar volume of cosolute, V_1, and 4) determine *m*-values and the PIP.

2.0 THEORY AND CONSEQUENCES OF PREFERENTIAL INTERACTION OF SOLUTION COMPONENT WITH A BIOMOLECULE

There are various forms of preferential interactions implied in the radial distribution function. They are water-water, solvent-solvent (in this case osmolyte), protein-water, protein-protein, and osmolyte-protein interactions. Interactions may be positive or negative. What Timasheff [9] called epithet, ‘‘preferential’’ refers to the relative affinities of the interacting loci on the protein for ligand and water. Using *C* as molarity symbol, the

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81 preferential hydration parameter (Γ_{21}) [14] and preferential osmolation parameter (Γ_{23}) [9]
82 can be given respectively as:

83 $$\Gamma_{21} = \left(\frac{\partial C_1}{\partial C_2}\right)_{T,P,\mu_1} = -\left(\frac{\partial \mu_2}{\partial \mu_1}\right)_{T,P,C_2} \quad (1)$$

84 $$\Gamma_{23} = \left(\frac{\partial C_3}{\partial C_2}\right)_{T,P,\mu_3} = -\left(\frac{\partial \mu_2}{\partial \mu_3}\right)_{T,P,C_2} \quad (2)$$

85 $$\Gamma_{23} = -\left(\frac{\partial \mu_3}{\partial C_2}\right)_{C_3} \Big/ \left(\frac{\partial \mu_3}{\partial C_3}\right)_{C_2} \quad (3)$$

86 Where μ_i stands for chemical potential of any solution components. The preceding
87 equations are in the furtherance of the reason why Γ_{2i} cannot be a measureable quantity
88 and a slope at the same time as previously reported [15]. According to Timasheff [9],

89 $$\Gamma_{21} = -\left({C_1}/{C_3}\right)\Gamma_{23} \quad (4a)$$

90 $$= \left({C_1}/{C_3}\right)\left(\frac{\partial \mu_3}{\partial C_2}\right)_{C_3} \Big/ \left(\frac{\partial \mu_3}{\partial C_3}\right)_{C_2} \quad (4b)$$

91 $$\Gamma_{23} = -\Gamma_{21}\left({C_3}/{C_1}\right) \quad (5a)$$

92 $$= \left(\frac{\partial \mu_2}{\partial \mu_1}\right)_{T,P,C_2}\left({C_3}/{C_1}\right) \quad (5b)$$

93 A close look at Eqs (4a) and (5a) shows that Γ_{23} cannot remain constant at different values
94 of C_3 and the latter is the only independent variable. The parameters, Γ_{23} and Γ_{21}, are
95 known to be measurable by biophysical methods such as dialysis equilibrium [9, 14],
96 sedimentation equilibrium [14], and pressure osmometry [9]. The change in Γ_{21} or Γ_{23} as
97 the case may seem to be, seems more important to the biochemist, pharmacist, and related

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98 specialist other than biophysicist. Such changes may compromise or inhibit the function of
99 the biomolecule as a result of conformational changes, the unfolding, partial folding and
100 dysfunctional rigidification that may arise depending on the kind of cosolvent and its
101 concentration. The change in Γ_{21} is directly related to the effect of water activity, a_1 or the
102 osmolyte osmotic pressure Π on the equilibrium constant $K_{\mathrm{eq}(1)}$ of the reaction which may
103 be conformational change [14].

104 $$\left(\frac{\partial \mathrm{In} K_{\mathrm{eq}(1)}}{\partial \mathrm{In} a_1}\right)_{\mathrm{T,P,C_2}} = -\frac{RT}{\bar{v}_1}\left(\frac{\partial \mathrm{In} K_{\mathrm{eq}(1)}}{\partial \Pi}\right)_{\mathrm{T,P,C_2}} = \Delta\Gamma_{21} \quad (6)$$

105 Where $R, T, P,$ and C_2 are gas constant, thermodynamic temperature, standard pressure,
106 and molarity of the biomolecule; $\bar{v}_1$ is the partial molar volume of water. Integrating the
107 derivative Eq. (6), gives the following.

108 $$\mathrm{In} K_{\mathrm{eq}(1)} = \Delta\Gamma_{21} \mathrm{In} a_1 \quad (7)$$

109 $$\mathrm{In} K_{\mathrm{eq}(1)} = -\frac{\bar{v}_1 \Pi}{RT} \Delta\Gamma_{21} \quad (8)$$

110 Timasheff [9] gives:

111 $$\mathrm{In} a_1 = C_3 \phi_3 / 55.56 = -\frac{\bar{v}_1 \Pi}{RT} \quad (9)$$

112 Where, the parameter ϕ_3 is the osmotic coefficient of the osmolyte.

113 The following equation may hold for preferential osmolation.

114 $$\Delta\Gamma_{23} = \left(\frac{\partial \mathrm{In} K_{\mathrm{eq}(3)}}{\partial \mathrm{In} a_3}\right)_{\mathrm{T,P,C_2}} \quad (10a)$$

115 Equation appears to be a slope against the backdrop of the fact that $\Delta\Gamma_{23}$ is also a
116 measureable parameter. This issue has been raised and concluded in favour of the view that
117 the parameter cannot be an instrument based measurable parameter and a slope at the
118 same time [15]. Thus, Eq. (10a) gives,

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119 $$\text{In}K_{\text{eq}(3)} = \Delta\Gamma_{23}\text{In}a_3 \quad (10b)$$

120 There are fundamental issues arising from Eq. (7), Eq. (8), and Eq. (10b). No devise
121 or equipment is known to measure $K_{\text{eq(i)}}$ directly. Rather absorbance of the biomolecule is
122 measured with variety of available biophysical equipment such as those for circular
123 dichroism spectroscopy, infrared spectroscopy, differential scanning calorimetry, Fourier
124 transform infrared spectroscopy etc [2]. These measurements can be taken at different
125 concentration of the osmolyte. The function of the biomolecule, enzyme for instance, may
126 also be monitored by taking the absorbance as a measure of the concentration of the
127 product of enzymatic action at different concentration of the osmolyte. Hence, the combined
128 biophysical model and biochemical model expressed via kinetic model. This issue will be
129 readdressed subsequently. It is not certain in literature, if the measuring device can measure
130 Γ_{23} and Γ_{21} simultaneously for every given concentration of the osmolyte. Devise such as
131 pressure osmometry is relevant to measurement of $\text{In}\left(\text{P}_1^{C_3}/\text{P}_1^0\right)$ or $\text{In}a_1$ [9] where, $\text{P}_1^{C_3}$ and P_1^0
132 are the vapour pressures of water for the solution of any osmolyte (or it may be protein,
133 whose concentration may be C_2) and water free from cosolute respectively.

134 Given the information implied in Eq. (11) above, a plot of $\text{In}K_{\text{eq(i)}}$ versus $\text{In}\boldsymbol{a}_1$ or
135 $\text{In}\boldsymbol{a}_3$ yields slopes, $\Delta\Gamma_{21}$ or $\Delta\Gamma_{23}$ respectively. However, if Γ_{23} is measured directly at 2
136 different values of C_3, then, $\Gamma_{23-2} - \Gamma_{23-1} \neq \Delta\Gamma_{23}$ where Γ_{23-2} and Γ_{23-1} are the Γ_{23}
137 values at higher and lower concentration of osmolyte respectively, if by definition, $\Delta\Gamma_{23}$ is
138 the slope as implied in Eq. (10b). It seems $\Delta\Gamma_{21}$ and $\Delta\Gamma_{23}$ may represent parameters
139 different from what they were meant to be. Meanwhile, a_3 and $a_1$are calculated after taking
140 measurement of relevant parameters. The parameter $K_{\text{eq}(3)}$ is also calculated after taking
141 measurement of needed parameters either by biophysical or biochemical methods. In other
142 words there are different values of $K_{\text{eq(i)}}$, a_3 or a_1 which are osmolyte concentration

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143 dependent. The ratio, $\frac{\text{In}K_{\text{eq(i)}}}{\text{In}a_{\text{i}}}$ gives value of $\Delta\Gamma_{2\text{ical}}$ (calculated value) that represents the

144 preferential interaction parameter at a defined C_3. This may be a mere speculation, the

145 essence of theoretical contribution. The parameter $\Delta\Gamma_{2\text{i}}$ as a slope may possess sign and

146 magnitude that merely reflects the degree of osmolation or hydration due to exclusion of

147 osmolyte. However, according to Timasheff [9], applying Eq. (4) gives, for the

148 calculated $\Delta\Gamma_{21}$, $\Delta\Gamma_{21\text{cal}} = -\left({C_1}/{C_3}\right)\frac{\text{In}K_{\text{eq(3)}}}{\text{In}a_3}$ and for the slope,

149

$$\Delta\Gamma_{21} = -\left({C_1}/{C_3}\right)\left(\frac{\partial\text{In}K_{\text{eq(3)}}}{\partial\text{In}a_3}\right)_{\text{P,T,C}_2}. \tag{12}$$

150 The implication of Eq. (12) is that there should be different values of $\Delta\Gamma_{21}$ for different C_3

151 because $\left(\frac{\partial\text{In}K}{\partial\text{In}a_1}\right)_{\text{P,T,C}_2}$ or $\Delta\Gamma_{23}$ is taken as slope and C_1 being molar concentration of water is

152 constant. Applying similar method to $\Delta\Gamma_{21}$ gives

153

$$\Delta\Gamma_{23} = -\left({C_3}/{C_1}\right)\left(\frac{\partial\text{In}K_{\text{eq(1)}}}{\partial\text{In}C_1}\right)_{\text{P,T,C}_2} \tag{13}$$

154 Another implication is that, $-\left({\text{C}_1}/{C_3}\right)\frac{\text{In}K_{\text{eq(3)}}}{\text{In}C_3} \neq -\left({C_1}/{C_3}\right)\left(\frac{\partial\text{In}K_{\text{eq(3)}}}{\partial\text{In}a_3}\right)_{\text{P,T,C}_2}$ and

155 $-\left({C_3}/{C_1}\right)\frac{\text{In}K_{\text{eq(1)}}}{\text{In}a_1} \neq -\left({C_3}/{C_1}\right)\left(\frac{\partial\text{In}K_{\text{eq(1)}}}{\partial\text{In}C_1}\right)_{\text{P,T,C}_2}$. This analysis confirms the earlier

156 suggestion that, the slopes may represent a parameter with meaning different from what it is

157 meant to be. This is against the backdrop of Wyman's equation known as the basic Wyman

158 linkage equation which, according to Timasheff [9], states that, "at any ligand concentration,

159 the gradient of the equilibrium constant with respect to ligand activity is equal to the change

160 in the binding of the ligand to the biological system during the course of the reaction (at

161 constant temperature and pressure that will be maintained throughout)". Nothing seems to

162 suggest that there is Wyman's equivalent equation for preferential hydration. The slope as

163 the change in the binding of the ligand may not give the same result of preferential exclusion

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164 according to Eq. (12). Besides, a measurable quantity such as $\Delta\Gamma_{2i}$ for the change or Γ_{2i} at
165 different finite concentrations of the osmolytes, extensive quantities, is also
166 thermodynamically an extensive quantity unlike a slope which is definitely an intensive
167 quantity under clearly specified conditions, temperature and pressure.

168 As explained elsewhere [15], another reason, why calculation of $\Delta\Gamma_{2i}$ may be more
169 useful for the determination of parameters is obtainable from the following equations [9, 14].
170 In their contributions, Shimizu [14] and Rösgen *et al* [3] attempted to relate preferential
171 interaction parameters with Kirkwood – Buff integrals (KBI). Beginning with Shimuzu [14] is
172 the equation:

173 $$\Gamma_{21} = N_{21} - \frac{C_1}{C_3} N_{23} \quad (14)$$

174 Where $C_\mathrm{i}(\text{or } n_\mathrm{i} \text{ as in literature})$ and $N_{2\mathrm{i}}$ represent respectively the density (molarity) of any
175 chemical species and the excess number of component *i* around the biomolecule, though
176 Eq. (14) is directly applicable to preferential hydration. The counterpart of Eq. (14) is the
177 osmolation case given as [9]:

178 $$\Gamma_{23} = N_{23} - \frac{C_3}{C_1} N_{21} \quad (15)$$

179 Equations (14) and (15) show that, the plot of measureable parameters versus either $1/C_3$ or
180 C_3 gives $C_1 N_{23}$ and N_{21}/C_1 respectively as slope. The equations for the change are given
181 as [9]

182 $$\left(\frac{\partial \text{In} K_{\text{eq}(1)}}{\partial \text{In} a_1}\right)_{\text{P,T,C}_2} = \Delta N_{21} - \frac{C_1}{C_3}\Delta N_{23} = \Delta\Gamma_{21} \quad (16)$$

183 $$\left(\frac{\partial \text{In} K_{\text{eq}(3)}}{\partial \text{In} a_3}\right)_{\text{P,T,C}_2} = \Delta N_{23} - \frac{C_3}{C_1}\Delta N_{21} = \Delta\Gamma_{23} \quad (17)$$

184 Before this time and recent publication [16], $K_{\text{eq}(3)}$ and $K_{\text{eq}(1)}$ are taken symbolically to be
185 K_{eq} which would have implied that $\partial \text{In} a_3 = \Delta\Gamma_{21} \partial \text{In} a_1 / \Delta\Gamma_{23}$. This is also quite different

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186 from $\Delta\Gamma_{21} = -\left({C_1}/{C_3}\right)\Delta\Gamma_{23}$. Perhaps it may not be intended to be so, but nothing in literature

187 tells the story on the contrary. If the parameter, $\Delta\Gamma_{2\mathrm{i}}$ from the plot of $\mathrm{In}K_{\mathrm{eq(i)}}$ versus $\mathrm{In}a_{\mathrm{i}}$

188 suggests that $\Delta N_{21} - \frac{C_1}{C_3}\Delta N_{23}$ or $\Delta N_{23} - \frac{C_3}{C_1}\Delta N_{21}$ is a slope then, as posited elsewhere [15]

189 a slope, such as $(\partial\mathrm{In}K/\partial\mathrm{In}a_1)_{\mathrm{P,T,C_2}}$ or $(\partial\mathrm{In}K/\partial\mathrm{In}a_3)_{\mathrm{P,T,C_2}}$ must not contain independent

190 variable such as C_3 given that molar concentration of water, C_1 is constant at a given

191 thermodynamic temperature.

192 **2.1 Examination of mathematical models connected to solution structure**

193 Solution structure involving the proteins can affect the function of the latter. Hence

194 the *m*-value need to be considered at all times. There are however, mathematical models or

195 equations that seem to create different forms of working equations when substituted into

196 initial equations, the derivative of the chemical potential of the osmolyte with respect to

197 osmolyte concentration. There is also relationship between the derivative of the chemical

198 potential of protein with respect to osmolyte concentration and the difference between

199 Kirkwood-Buff integral (KBI) for hydration and KBI for osmolation [3]. In this protein related

200 issue, the mathematical equations which appear in the derivatives lead to what seems to be

201 inconsistent equations. Because of the central role of *m*-value, it is reviewed here before,

202 examination of mathematical equations that affects its derivation.

203 The extent to which the interaction of different osmolytes may cause changes in the

204 structure and function of proteins in particular may differ. To Poklar *et al*. [17], the physical

205 significance of the factor, *m*-value, is not completely clear despite its wide spread use in

206 recent time, though it has been viewed as the difference in the amount of the denaturant

207 interacting with the native and denatured states of the polypeptide chain [17]. As stated

208 elsewhere [18], if *C*½ represent the concentration of the osmolyte needed to cause

209 denaturation of half the given protein concentration then high *m*-value and low *C*½ values

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210 indicate high effectiveness of a given denaturant [17]. Similar definition may be applicable to
211 an osmolyte that can force folding.

212 Once again the *m*-value is a measure of the effect of an osmolyte on protein
213 stability. It is the slope $(m - \text{value} = \text{dG}/\text{d}C_3)$ of a plot of the native to denatured free
214 energy change as a function of osmolyte concentration (C_3). This is the opinion of Marcelo *et*
215 *al* [19] and as cited by Harries and Rösgen. [20]. The *m*-value is a reflection of the effect that
216 a change in the concentration of the osmolyte (co-solute) has on the stability of the protein
217 and it is a good measure of the effectiveness of the osmolyte's ability to force the protein
218 either to fold or unfold. Meanwhile, the preferential interaction can also be used as an
219 alternative descriptor for the $m - value$ [20]. This is to say that there could be a link
220 between preferential interaction parameter and $m - \text{value}$. This can be achieved via the KBI
221 as indicated by Rösgen *et al* [3], although with reservation due to what seems to be a
222 mathematical mistake or perhaps, misconception in an effort to define the structural basis for
223 the $m - \text{value}$ as found in literature [3].

224 In this research the slope, $\left(-\left(\frac{\partial \text{In}K_{\text{eq}(3)}}{\partial \text{C}_3}\right)_{\text{T,P}} = \frac{m}{RT}\right)$ [7], whose magnitude and sign
225 indicate the capacity of the osmolyte to (re) fold or unfold a protein is adopted. In this regard,
226 the protecting osmolyte has positive $m - \text{value}$ while a destabilising osmolyte has a
227 negative $m - \text{value}$ [7].

228 Mathematically the structural basis for the $m - \text{value}$ is according to Rösgen *et al*
229 [3] given as

230 $$-\left(\frac{\partial \text{InK}}{\partial \text{C}_3}\right)_{\text{T,P}} = \frac{m}{RT} = \frac{\Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})}{1 - C_3(G_{13} - G_{33},)} \quad (18)$$

231 Where, $G_{13} - G_{33}$, and G_{13} and G_{33} are the apparent hydrated molar volume of the
232 osmolyte, KBI for osmolyte hydration and osmolyte self osmolation (correlation) respectively;
233 G_{21} and G_{23} are respectively the KB integral for hydration and osmolation of the protein. The

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234 issue in contention is about the equation [3] which perhaps is mistakenly given as

235 $$\frac{1}{1-C_3(G_{13}-G_{33})} = \frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \quad (19a)$$

236 Equation (19a) has issue with dimension if the unit (L/mol) of $G_{13}-G_{33}$ is taken into

237 account. Besides, if $\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}}$ is taken as slope, any calculation to obtain $(G_{13}-G_{33})$, leads

238 to highly contentious result. Nonetheless, it is to be substituted into all relevant equations to

239 enable the verification of any claim regarding the invalidity of whatever equations that arise

240 in this research as well as in literature. However, there is a need to point out the fact

241 that C_3 is the same at the left - and right - hand sides of Eq. (19a); but the introduction of

242 standard-state molarity given as $C_i = C_i^0 = 1\ \mathrm{mol/L}$ at the right-hand side corrects the

243 dimensional inconsistency. The corollary is that there should be the expression given as

244 $a_i = a_i^0 = 1\ \mathrm{mol/L}$. Thus Eq. (19a) can be rewritten as $\frac{1}{1-C_3(G_{13}-G_{33})} = \frac{1}{RT/C_3^0}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}}$,

245 thereby eliminating dimensional inconsistency. According to Rösgen *et al* [3], the derivative

246 is given as

247 $$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} = \frac{1}{C_3} + \frac{G_{13}-G_{33}}{1-C_3(G_{13}-G_{33})} \quad (19b)$$

248 It is important to realise that the denominator at the left hand side also appears in the

249 derivative relating the chemical potential of the protein to the osmolyte concentration and to

250 the KBI for the hydration and osmolation of protein. This is given for the protein as follows

251 [3].

252 $$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{\mathrm{T,P}} = \frac{G_{21}-G_{23}}{1-C_3(G_{13}-G_{33})} \quad (19c)$$

253 Henceforth, $G_{13}-G_{33}$ is designated as V_1, the apparent hydrated molar volume of the

254 osmolyte. If Eq. (19a) is substituted into Eq. (19b) one obtains

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255 $$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} = \frac{1}{C_3} + \frac{V_1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \quad (20)$$

256 Rearrangement followed by integration gives

257 $$\Delta\mu_3 = \frac{RT}{1-V_1}\mathrm{In}C_3 \quad (21)$$

258 None of these equations, Eq. (20) and Eq. (21) can be valid because the dimension or unit
259 of final result is incorrect just like the result from the original equation, Eq. (19a). If
260 thermodynamic principle is valid, then, for an ideal solution, $\Delta\mu_3 = RT\mathrm{In}C_3$. This makes the
261 denominator in Eq. (21) irrelevant. But under such ideal condition, $V_1 = 0$ thereby,
262 confirming the issue of relevance or validity. However, the ideal situation does not give
263 absolute equality between C_3 and a_3; this implies that, though $\Delta\mu_3 \cong RT\mathrm{In}(C_3)$,
264 nevertheless, the difference may be important in the determination of V_1 in Eq. (21). It is
265 important noting is taken for granted. But that is not all because if ideality is precluded, the
266 issue of dimensional inaccuracy cannot be precluded.

267 If Eq. (19a) is substituted into Eq. (19c) one obtains

268 $$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21}-G_{23}}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \quad (22a)$$

269 Rearrangement gives

270 $$\left(\frac{\partial \mu_2}{\partial \mu_3}\right)_{T,P} = G_{21} - G_{23} = -\frac{\Gamma_{23}}{C_3} \quad (22b)$$

271 The denominator, C_3 in Eq. (22b) makes the latter different from Eq. (2) [2]. Substitution of
272 Eq. (19a) into Eq. (18) gives

273 $$m = \Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21} - G_{23})\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \quad (23)$$

274 Equation (23) like any other equation arising from the use of Eq. (19a), is dimensionally
275 inaccurate.

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276 On the other hand, Eq. (19a) may be rewritten as

277 $$\frac{1}{1-C_3(G_{13}-G_{33})} = \frac{1}{RT}\left(\frac{\partial \mu_3}{\partial \text{In}C_3}\right)_{T,P} \quad (24)$$

278 In the paper by Rösgen *et al* [3] $\left(\frac{\partial \mu_3}{\partial \text{In}C_3}\right)_{T,P}$ was used in the determination of the structural

279 basis of the *m*-value (*m* for short), which is, seemly suggestive of an initial technical error.

280 There is no issue of dimensional inaccuracy in Eq. (24) if $\text{In}C_3$ is rewritten as $\text{In}(C_3/C_3^0)$. But

281 the independent parameter cannot appear as a constant and as a variable considering the

282 partial differential $\partial\text{In}(C_3/C_3^0)$ even if $\left(\frac{\partial \mu_3}{\partial \text{In}(C_3/C_3^0).}\right)_{T,P}$ is taken as slope. However, the

283 continuous appearance of C_3 in the equations, demands examination shortly. Before this,

284 there is need to realise that $\left(\partial \mu_3/\partial \text{In}C_3\right)_{T,P,C_3=0} = RT$ if $C_3 \rightarrow 0$ (i.e. a case of infinite

285 dilution). This seems to be the valid view of Rösgen *et al* [3]. If this is the case most of the

286 preceding equations where C_3, instead of $\text{In}C_3$, appears cannot be valid. The implication is

287 that $G_{13} - G_{33} = 0$ $(G_{13} = G_{33})$. However, in subsequent derivations, C_3 is regarded as one

288 which is » 0. But before this, the issue regarding ideality is reexamined as follows.

289 Substitution of Eq. (24) into Eq. (19b) gives

290 $$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{T,P} = \frac{1}{C_3} + \frac{V_1}{RT}\left(\frac{\partial \mu_3}{\partial \text{In}C_3}\right)_{\text{T,P}} \quad (25)$$

291 Rearrangement and integration gives (note that, $\partial \mu_3/\partial \text{In}C_3 = RT$) for an ideal case

292 $$\frac{\Delta \mu_3}{RT} = \text{In}C_3 + \Delta C_3 V_1 \quad (26)$$

293 But $\frac{\Delta \mu_3}{RT} = \text{In}C_3$ (or more appropriately, $\text{In}(C_3/C_3^0)$ for an ideal case, such that, $\Delta C_3 V_1 = 0$:

294 This is as often stated in literature [3]. What the value of ΔC_3 should be needs to be

295 ascertained. One cannot shy away from the fact that the adoption of standard-state molarity

296 implies a transition from 1 mol/L to values of $C_3 \ll 1\ \text{mol/L}$ or > 1 mol/L as the case may be.

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297 But as stated earlier, the infinitesimal difference between C_3 and a_3 may be useful for the
298 determination of V_1. In such situations, the value of V_1 obtained by calculation may be
299 negative if activity coefficient is < 1 mol/L. Ideal case is to be applied to dilute solution of the
300 protein as follows. Substitution of Eq. (24) into Eq. (19c) gives

301 $$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21}-G_{23}}{RT}\left(\frac{\partial \mu_3}{\partial \mathrm{In} C_3}\right)_{\mathrm{T,P}} \quad (27a)$$

302 $$\iint \partial\, \mathrm{In} C_3 . \left(\partial \mu_2\right)_{\mathrm{T,P}} = (G_{21} - G_{23}). \iint \partial \mu_3 . \partial \mathrm{C}_3 \quad (27b)$$

303 Rearrangement of Eq. (27a) and integration as shown in Eq. (27b) gives

304 $$\mathrm{In} C_3 . \left(\Delta \mu_2\right)_{\mathrm{T,P}} = (G_{21} - G_{23}) \Delta \mu_3 . \Delta C_3 \quad (27c)$$

305 Once again if standard state molarity is taken into account, then $\mathrm{In} C_3 - \mathrm{In} C_3^0 = \mathrm{In} C_3$: the
306 question is, what is the expression for the change in [C_i] if it cannot be defined by $\Delta C_3 =$
307 $C_3 - C_3^0$? Therefore, for the ideal case,

308 $$\left(\Delta \mu_2\right)_{\mathrm{T,P}} = RT \Delta C_3 (G_{21} - G_{23}) \quad (28a)$$

309 If in Eq. (28a), $\left(\Delta \mu_2\right)_{\mathrm{T,P}}/\Delta C_3$ $(\mathrm{or}\ \partial \mu_2/\partial C_3)$ is taken as slope from the plot of $\left(\Delta \mu_2\right)_{\mathrm{T,P}}$
310 versus C_3, the difference between the KBI for hydration of protein and KBI for its osmolation,
311 $G_{21} - G_{23}$ should be equal to slope/RT or $\left(\left(\Delta \mu_2\right)_{\mathrm{T,P}}/\Delta C_3/RT\right)$. Considering
312 that $C_3(G_{21} - G_{23}) = -\Gamma_{23}$ then, the following equation may be applicable.

313 $$C_3 \left(\Delta \mu_2\right)_{\mathrm{T,P}}/\Delta C_3/RT = -\ \Gamma_{23} \quad (28b)$$

314 The chemical potential of the protein (enzyme) can be determined if the concentration of
315 unfolded enzyme is known; the fraction of the total concentration of the cosolute-treated
316 enzyme which is equal to the concentration of the unfolded protein multiplied by total
317 concentration of the enzyme can be used to determine $\left(\Delta \mu_2\right)_{\mathrm{T,P}}$.

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318 Looking at Eq. (28b) one sees that the chemical potential of the protein can either be
319 positive or negative if respectively, the preferential interaction parameter by exclusion or
320 binding is the case. Eq. (28b) represents a precedence whose validity or scientific merit
321 remains a matter for feature investigation. Considering that the concentration (ranging from
322 nanoscale−milli−scale mol/L) of the enzyme is very low in most laboratory/clinical
323 investigation, one can correctly admit that ideality should be the case: One may need to
324 recall that Eq. (28b) is an outcome of contentious equations, namely Eq. (19a) and Eq. (24).

325 In terms of structural basis for the $m-\text{value}$

326 $$m = \Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})RT \tag{29a}$$

327 $$m = -RT\Delta_{\text{N}}^{\text{D}}\Gamma_{23}/\,C_3 \tag{29b}$$

328 It seems that with respect to the $m-\text{value}$, the place of ideality may not be ruled out
329 probably on account of the fact that $\text{In}\left(1/K_{\text{eq}}\right)$ is plotted versus C_3. With the end of the
330 consideration for ideal situation, subsequent derivations take into account nonideal cases.
331 This was implied in previous research [15] but it was not explicitly stated.

332 The nonideal case is hereby considered beginning with the dependence of the
333 osmolyte's chemical potential on the osmolyte concentration. Rearrangement of Eq. (25) for
334 integration gives

335 $$\frac{1}{RT}\iint \partial\,\text{In}C_3.\left(\partial\mu_3\right)_{\text{T,P}} = \iint \frac{\partial C_3}{C_3}\partial\text{In}C_3 + \iint \frac{V_1\partial\mu_3(\partial C_3)_{\text{T,P}}}{RT} \tag{30a}$$

336 But in the light of other parameters that need to be determined, InC_3 should be replaced by
337 Ina_3 for the nonideal case (N.B. $\Delta\mu_3 = RT\text{In}a_3$). Rearrangement and integration of Eq. (25)
338 as shown in Eq. (30a) gives

339 $$\frac{1}{RT}\Delta\mu_3\text{In}a_3 = (\text{In}a_3)^2 + \frac{V_1\Delta\mu_3\Delta C_3}{RT} \tag{30b}$$

340 If V_1 is known, then the chemical potential of the osmolyte is given as

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341 $$\Delta\mu_3 = \frac{(\text{In}a_3)^2}{\left(\frac{\text{In}a_3}{RT} - \frac{V_1 \Delta C_3}{RT}\right)} \quad (30c)$$

342 $$V_1 = \frac{\text{In}a_3.\text{In}a_3 - (\text{In}a_3)^2}{\Delta C_3.\text{In}a_3} \quad (31)$$

343 A closer view of Eq. (31) should reveal that after substituting relevant parameters into it, the
344 calculable value of V_1 is equal to zero. This situation may not be suitable for the
345 determination of the Kirkwood-Buff integral for hydration and osmolation. The dependence of
346 chemical potential of dilute protein on the osmolyte concentration (for nonideal case) initially
347 given in Eq. (27c) is restated as (N.B. In Eq. (27c), $\Delta\mu_3 = RT\text{In}a_3$)

348 $$(\Delta\mu_2)_{\text{T,P}} = \frac{(G_{21} - G_{23})\Delta C_3 RT\text{In}a_3}{\text{In}a_3} \quad (32a)$$

349 $$= (G_{21} - G_{23})\Delta C_3 RT \quad (32b)$$

350 In the light of the Eq. (2) [9], there is need to revisit Eq. (27a). Rearranging the latter gives

351 $$\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{\text{T,P}} = \frac{(G_{21} - G_{23})RT}{RT}\left(\frac{\partial C_3}{\partial\text{In}C_3}\right)_{\text{T,P}} \quad (33a)$$

352 $$= (G_{21} - G_{23})\left(\frac{\partial C_3}{\partial\text{In}C_3}\right)_{\text{T,P}} \quad (33b)$$

353 The implication is that

354 $$\left(\frac{\partial C_3}{\partial C_2}\right)_{\text{T,P},\mu_3} = -(G_{21} - G_{23})\left(\frac{\partial C_3}{\partial\text{In}C_3}\right)_{\text{T,P}} \quad (33c)$$

355 Rearrangement of Eq. (33c) gives

356 $$(\partial C_2)_{\text{T,P},\,\mu_3} = -\,\partial\text{In}C_3/(G_{21} - G_{23}) \quad (33d)$$

357 Looking at Eq. (33d), one sees that $(\partial C_2)_{\text{T,P},\,\mu_3}/\,\partial\text{In}C_3$ looks like a slope, appropriately
358 from the plot of C_2 versus $\text{In}(C_3/C_3^0)$. Therefore, it may not be out of place to rewrite Eq.
359 (33d) as follows:

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360 $$C_2 = -\text{In}(C_3/C_3^0)/(G_{21} - G_{23}) \quad (33e)$$

361 Due to the effect and the presence of a cosolute, there may be the occurrence of a
362 preponderance of either the unfolded or (re)folded enzyme such that a plot of the
363 concentration of (un)folded versus (C_3/C_3^0) gives a slope equal to $1/(G_{21} - G_{23})$; this
364 remains conjecturally possible.

365 The nonideal case for the determination of the structural basis of the *m*-value is
366 given by rewriting Eq. (23); instead of InC_3, Ina_3 is used as follows.

367 $$m = \Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})\left(\frac{\partial\mu_3}{\partial\text{In}a_3}\right)_{\text{T,P}} = \Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})RT\left(\frac{\partial\text{In}a_3}{\partial\text{In}a_3}\right)_{\text{T,P}} \quad (34a)$$

368 $$= \frac{-RT\Delta_{\text{N}}^{\text{D}}\Gamma_{23}}{C_3}\left(\frac{\partial\text{In}a_3}{\partial\text{In}a_3}\right)_{\text{T,P}} \quad (34b)$$

369 $$\frac{C_3 m}{RT} = -\Delta_{\text{N}}^{\text{D}}\Gamma_{23} \quad (34c)$$

370 Looking closely at Eq. (34a) and Eq. (34b), it would appear that there are 3 slopes
371 viz: $\Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23}), m - \text{value}'$ and $\Delta_{\text{N}}^{\text{D}}\Gamma_{23}$. If the values of $G_{21}(= \Delta N_{21}/C_1)$ and $G_{23}(=$
372 $\Delta N_{23}/C_3)$ are obtained from the plot of $\Delta\Gamma_{2\text{i}}$ versus C_3 or $1/C_3$, as the case may be,
373 according to Eq. (17) and Eq. (16) respectively then, $\Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})$ may speculatively be
374 taken as a constant or slope. Therefore, $\Delta_{\text{N}}^{\text{D}}\Gamma_{23}$ can be calculated for different values of C_3,
375 thereby justifying the claim that the former cannot be a constant quantity or slope and
376 equipment based measurable parameter. It is definitely obvious that $a_{\text{i}} \neq C_3$ and as such a
377 plot of $\text{In}a_3$ versus $\text{In}C_3$ cannot be equal to one even if the coefficient of determination may
378 be one. An equation relating $\text{In}a_3$ to $\text{In}C_3$ may be expressed as: $\text{In}a_3 = \text{Ş}\text{In}C_3 - \text{Í}$ where Ş
379 and Í are the slope and intercept respectively**.** However, this is not to justify the place of
380 $\frac{\partial\text{In}a_3}{\partial\text{In}C_3}$ or $\left(\frac{\partial\mu_3}{\partial\text{In}C_3}\right)_{\text{T,P}}$. Previous publication [15] and, as pointed out earlier in the text, has

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381 strongly shown that all except $m - \text{value}$ are not slope and consequently they are extensive

382 quantity; the other two, $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$ and Γ_{23} cannot be a devise based measurable parameter

383 and constant quantities at the same time. In previous research [15] the change in solvation

384 preference upon unfolding in terms of the *m*-value equation was determined by eliminating

385 the apparent hydrated molar volume of the osmolyte. But if V_1 is relevant and correctly

386 known, it may be used to calculate the same parameter at different values of C_3. Thus,

387 $$\mu_3 = \mu_3^0 + RT\mathrm{In}\left(\frac{C_3}{1-V_1C_3}\right) \quad (35a)$$

388 Equation (35a) is obtained by integrating the derivative (Eq. (19b)) given by KB theory [3]

389 with respect to C_3 while holding V_1 constant. Rearrangement of Eq. (35a) gives

390 $$\frac{\Delta\mu_3}{RT} = \mathrm{In}\left(\frac{C_3}{1-V_1C_3}\right) \quad (35b)$$

391 **2.2.0 Apparent hydrated molar volume, a variable or a constant?**

392 Here apparent hydrated molar volume of cosolutes is to be determined based on

393 different principles. There are arguments about the validity of derived equations based on

394 fundamental equations and recent equations in this research.

395 **2.2.1 Determination based on the presumed relationship with activity coefficient.**

396 In line with Timasheff equation [9] but on the basis of molar concentration,

397 $$\frac{\Delta\mu_3}{RT} = \mathrm{In}C_3\gamma_3 = \mathrm{In}\left(\frac{C_3}{1-V_1C_3}\right) \quad (36a)$$

398 Where, the parameter γ_3 is the osmolyte activity coefficient. Although the standard reference

399 concentration can be introduced into Eq. (36a), its presence both at the right - and left - hand

400 sides makes it unnecessary.

401 $$C_3\gamma_3 = \frac{C_3}{1-V_1C_3} \quad (36b)$$

402 $$\gamma_3 = \frac{1}{(1-V_1C_3)} \quad (36c)$$

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403 One advantage of Eq. (36a or 36b) is that, *ab initio*, there is no dimensional issue, pointing to
404 a probable validity. In order to determine V_1 graphically, Eq. (36c) can be transformed into,
405 first,

406
$$\frac{1}{\gamma_3 V_1} = \frac{1}{V_1} - C_3 \tag{37a}$$

407 Rearrangement of Eq. (37a) gives

408
$$\frac{\gamma_3 - 1}{\gamma_3} = V_1 C_3 \tag{37b}$$

409 A plot of $\frac{\gamma_3 - 1}{\gamma_3}$ versus C_3 gives a positive slope with increasing γ_3 and, if $\gamma_3 < 1$ the
410 calculated values should be negative in sign. This raises question as to the validity of V_1 if it
411 must always be a positive quantity. The issue of validity is strongly applicable to Eq. (31).
412 The values of V_1 can also be determined directly from Eq. (35b) and Eq. (37b); the values
413 obtainable may be slightly higher than those obtainable from Eq. (31). This is not to support
414 the negative value of V_1, a parameter that differs for different values of C_3.

415 3.0 MATERIALS AND METHODS

416 3.1 Materials

417 The chemicals used were: The chemicals used were: Sucrose (St Lious France); raw
418 (native) potato starch (Sigma Chemicals Co, USA); ethanol, hydrochloric acid and sodium
419 chloride (BDH Chemical Ltd, Poole England); 3,5-dinitrosalicylic acid (DNA) (Lab Tech
420 Chemicals, India); Tris (Kiran Light Laboratories, USA); porcine pancreatic alpha amylase (EC
421 3.2.1.1) (Sigma, Adrich, USA); all other chemicals were of analytical grade and solutions were
422 made in distilled water. Aspirin was purchased from CP Pharmaceuticals Ltd, Ash road North,
423 Wrexham, LL 13 9UF, U.K.

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427 ### 3.2 Equipment

428 *pH* meter (tester) from Hanna Instruments, Mauritius; electronic weighing machine
429 from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 300D model from China; 721/722
430 visible spectrophotometer from Spectrum Instruments Co Ltd, China.

431 ### 3.3 Methods

432 Bernfeld method [21] of enzyme assay was adopted for the assay of the enzyme,
433 porcine pancreatic alpha amylase (PAA). Preparation of substrate and enzyme was as
434 described elsewhere [16]. Equilibrium constant for folded to unfolded transition is either
435 according to Eq. (49) or Eq. (53) as the case may be. The calculation of preferential
436 interaction parameter for folded to unfolded transition is according to Eq. (34c or 29b). Plots
437 for the determination apparent hydrated molar volume, are according to Eq. (37b), Eq. (40b)
438 and E. (44) (Eq. (40b) and E. (44) are in the supplementary section). Determination of
439 thermodynamic activity of solvent and solute and corresponding activity coefficient was as
440 described elsewhere [16]. Microsoft Excel (2007) was used to plot the dependent variable
441 versus independent variable.

442 ### 3.4 Statistical analysis

443 The velocities of hydrolysis were determined in triplicates. The mean values were
444 used to determine the equilibrium constant for folded to unfolded protein transition.

445 ## 4. RESULTS AND DISCUSSION

446 The important purpose of the theoretical section, a major part of this research is to
447 proffer a proper basis of any interpretation of results obtained from the changes of the
448 biomolecular function. Such change may result from change of structure due to solution
449 composition. It is very imperative that mathematical models or equations used to qualitatively
450 and in most cases quantitatively interpret results are valid. Thus as was observed in the
451 theoretical section, the appearance of $RT\text{In}C_3$ gives the impression of ideality. This leads to
452 a situation where the apparent hydrated molar volume, V_1 of the osmolyte is equal to zero.
453 The continuous use of $RT\ \text{In}C_3$ demands that C_3, though low, must be much greater than 0.

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The different calculated values of V_1 are shown in Table 1. This is applicable to Eq. (31), Eq. (37b), and Eq. (40b). Mathematically and from the standpoint of dimensionality in particular, equations that are not valid are Eq. (20)-Eq. (23). Equations that appear valid from the same stand point due to the substitution of Eq. (24) which appears dimensionally valid are Eq. (25) to Eq. (30c). But this is mainly a dimensionality issue whose validity validates in part the mathematical models or equations. Thus beyond dimensional validity, substitution of Eq. (24) into a particular equation does not always produce a valid equation as observed in this research. This is applicable to Eq. (33a-33d), where there is need to introduce the standard reference concentration equal to 1 mol/L.

The slopes (see Figures 1a-1d, 2a-2d, & 3a-3d) for all are positive but unlike the slope from plot based on Eq. (37b) the slopes from plots based on Eq. (40b) and Eq. (44) are very high in magnitude (Table 1). The plots where the data are generated are shown as Figures 1a -1d, 2a-2d, and 3a-3d respectively. This is strictly for the purpose of illustration; the order of magnitude is Eq. (37b) < Eq. (40b) < Eq. (44).

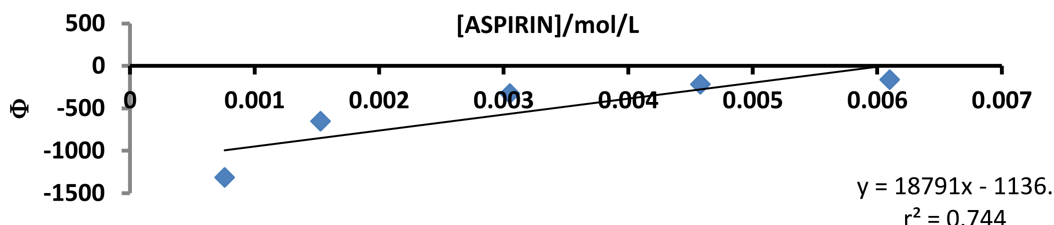

Fig. 1a. A plot for the determination of apparent hydrated molar volume as function of *[Aspirin].* *The symbol Φ stands for 1-(1/a_3). Note that the coefficient of determination r^2 (0.744<0.900) expresses nonlinearity.*

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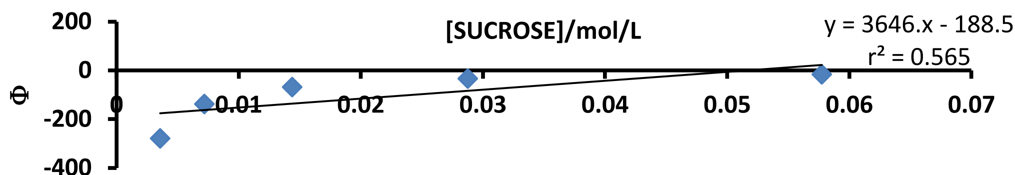
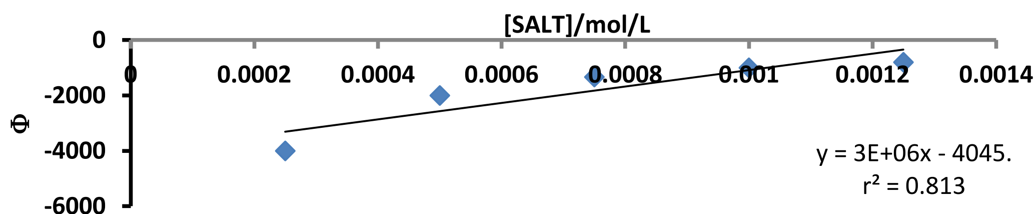
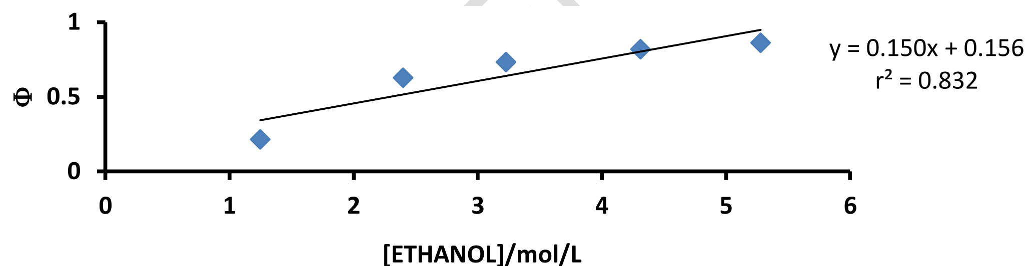


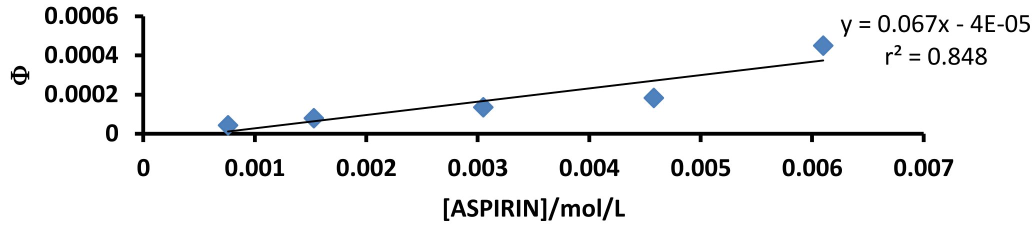
477
478
479 **Fig. 1b. A plot for the determination of apparent hydrated molar volume as function of**
480 **[Sucrose].** *The symbol Φ stands for 1-(1/a_3). The coefficient of determination r^2 (0.565 <*
481 *0.900) expresses nonlinearity.*
482



483
484
485 **Fig. 1c. A plot for the determination of apparent hydrated molar volume as function of**
486 ***[Salt].*** *The symbol Φ stands for 1-(1/a_3).The salt is calcium chloride. Note that the coefficient*
487 *of determination r^2 (0.813 < 0.900) expresses nonlinearity.*
488

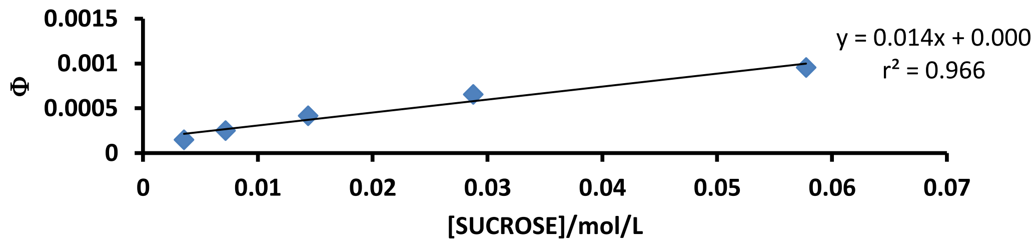


489
490
491 **Fig. 1d. A plot for the determination of apparent hydrated molar volume as function of**
492 **[Ethanol].** *The symbol Φ stands for 1-(1/a_3). The coefficient of determination r^2 (0.832 <*
493 *0.900) expresses nonlinearity.*
494
495
496
497
498
499

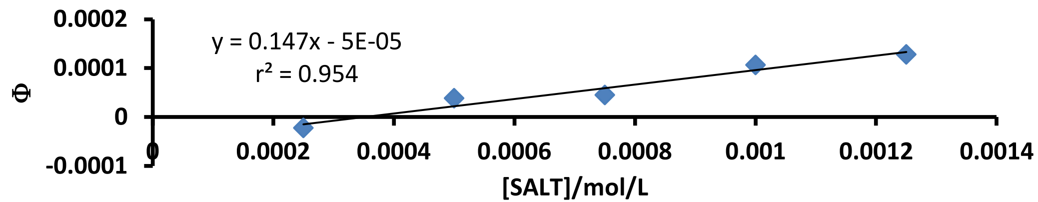


500

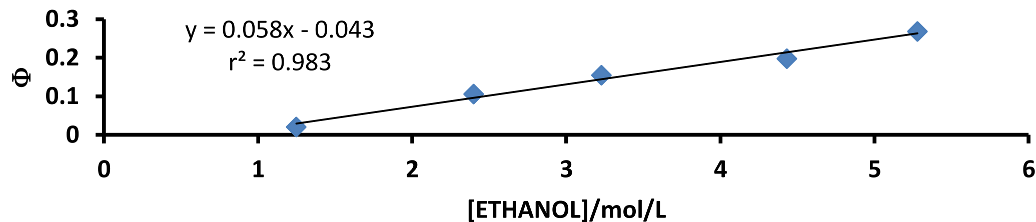
501 **Fig. 2a. A plot for the determination of apparent hydrated molar volume as function of**
502 **molar concentration of [Aspirin];** *the parameter Φ is $1-1/\gamma$. The coefficient of*
503 *determination r^2 (0.848 < 0.900) expresses nonlinearity.*



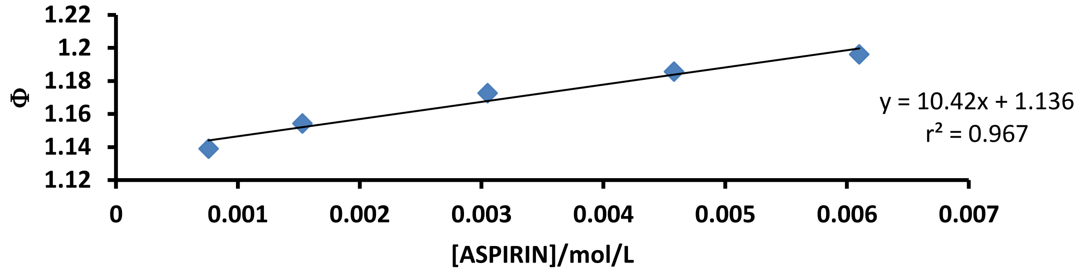
504
505
506 **Fig. 2b. A plot for the determination of apparent hydrated molar volume as function of**
507 **molar concentration of [Sucrose];** *the parameter Φ is $1-1/\gamma$.*
508
509



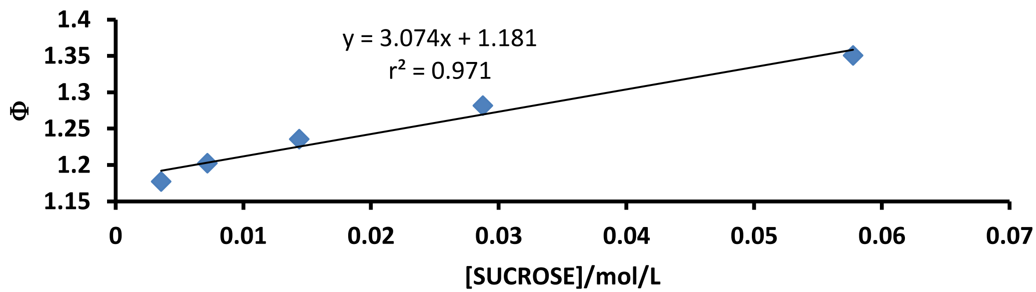
510
511
512 **Fig. 2c. A plot for the determination of apparent hydrated molar volume as function of**
513 **molar concentration of [Salt];** *the parameter Φ is $1-1/\gamma$.*
514



515
516 **Fig. 2d. A plot for the determination of apparent hydrated molar volume as function of**
517 **molar concentration of [Ethanol];** *the parameter Φ is $1-1/\gamma$.*
518

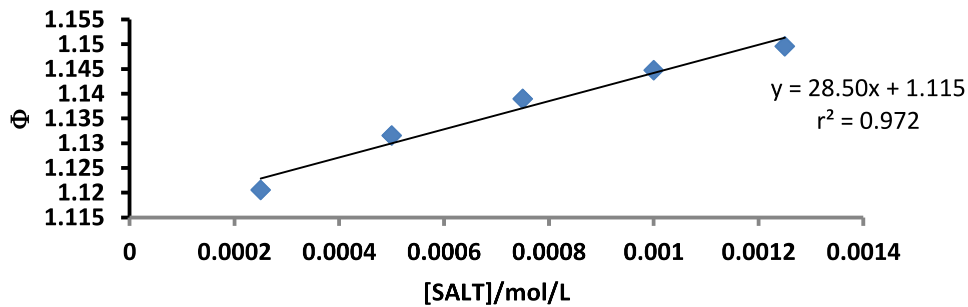


519
520
521 **Fig. 3a. A plot for the determination of apparent hydrated molar volume as function of**
522 **[Aspirin]**; *the parameter Φ is 1−1/lna$_3$.*
523



524

525 **Fig. 3b. A plot for the determination of apparent hydrated molar volume as function of**
526 **[Sucrose];** *the parameter Φ is 1−1/lna$_3$.*
527



528

529 **Fig. 3c. A plot for the determination of apparent hydrated molar volume as function of**
530 **[Salt];** *the parameter Φ is 1−1/lna$_3$.*
531

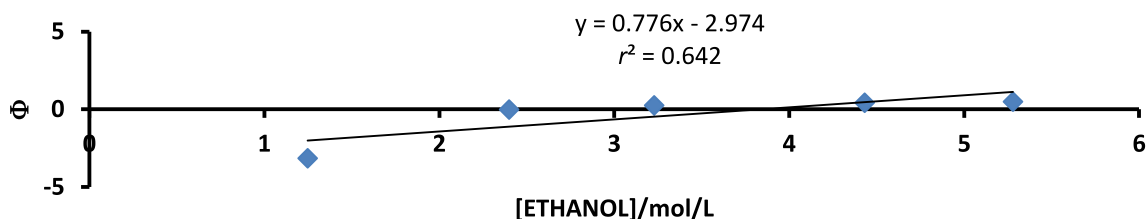


Fig. 3d. A plot for the determination of apparent hydrated molar volume as function of [Ethanol]; *the parameter Φ is 1−1/lna$_3$. The coefficient of determination r^2 (0.642 < 0.900) expresses nonlinearity.*

Of particular note is the observed similar values obtained for ethanol based on Eq. (40b) and Eq. (44) (Table 1). This goes to show that concentration regimes seem to create different slopes and, most importantly the derived mathematical equations may not be appropriate unlike Eq. (37b). The values of V_1 based on Eq. (37b) can better serve calculational purpose that gives positive result of other parameters when substituted into relevant equations in literature [3]. For instance, but for the feature, the values based on Eq. (37b) can be used to determine the change of solvation preference (this is given as $\Delta_N^D(G_{21} - G_{23})$) upon denaturation if the *m*-value is known. It can also be used to determine the chemical potential of osmolyte ($\Delta\mu_3$) given as $RT\text{In}\,(C_3/C_3^0)/(1 - C_3V_1)\,G_{23}$, the modified form of Rösgen *et al* [3'] equation. It needs to be stated that this approach is slightly different from conventional methods in literature [22], though it seems to enable the determination of V_1 if $\Delta\mu_3$ is independently determined.

Table 1. Determination of apparent hydrated molar volumes of cosolutes

Equations	[Ethanol]	[Salt]	[Sucrose]	[Aspirin]
		V_1		
Eq. (37b)	0.060	0.147	0.014	0.067
r^2	0.995	0.954	0.966	0.847
Eq. (40b)	0.148	28.500	3.076	10.42
r^2	0.831	0.972	0.970	0.968
Eq. (44)	0.150	3.000 E	3.646 F	18.918 F

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565 r^2 0.832 0.813 0.566 0.749
566
567 *The parameter V_1 is the apparent hydrated molar volume of cosolutes. The coefficient of*
568 *determination (r^2) is indicated so as to emphasise the departure from linearity where*
569 *applicable rather than only the occurrence of outliers arising from imperfection in the assay.*
570 *E means exp (+6); F means exp (+3).*
571

572 The capacity of cosolute to force refolding or unfolding, the *m*-value was determined
573 either with a single or multiple cosolute. With ethanol alone unlike with a mixture of the
574 former and sucrose, the *m*-value was positive in sign (Table 2a). With respect to ethanol
575 alone, the positive *m*-value is similar to the result achieved in the past [16]. There has been
576 report that an organic solvent which should have been destabilising may become a stabiliser
577 [23]. To this end, "low water – content ethanol is preferentially excluded from the protein
578 surface" [23]. If this is the case, there may have been positive *m*-value for such solvent,
579 ethanol as in this research. However, the interest in this research is to use alternative
580 equation to determine the preferential parameters via Eq. (29c) and Eq. (34c).

581
582 **Table 2a. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
583 **with the enzyme, in a reaction mixture, containing sucrose and ethanol-RTln$K_{eq(3)}$ as a**
584 **function of [Ethanol] (1.25, 2.40, 3.23, 4.31, 5.28 mol/L).**
585

586 [Sucrose] 587 (mmol/L)	0.00	3.57	7.19	14.38	28.76	57.75
588						
589 *m*-value 590 (kJL/mol^2)	+1.60	–1.78	– 3.03	– 1.67	– 0.69	–0.44
591						
592 r^2	0.86	0.97	0.93	0.94	0.99	1.00^z

593
594 *The data is obtained from the plot of In1/$K_{eq(3)}$ versus [Ethanol] with different concentration of*
595 *sucrose; the superscript z indicates datum from a straight line of two-data points; (r^2) is the*
596 *coefficient of determination.*
597

598 The fact that there were negative *m*-values with a mixture of ethanol and sucrose,
599 points to the possibility that sucrose may either have reduced the solubility of water insoluble
600 native potato starch or has reduced the conformational flexibility of the enzyme needed for
601 function. According to Kurkal *et al* [24] proteins, dynamics otherwise called 'loosening up'
602 facilitates biological function of enzymes. In the same vein, according to Affleck *et al* [25] the

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603 increased conformational flexibility due in part, to the reduced interaction of charged and /or
604 polar amino acid residues within the enzyme molecules is caused by water's ability to effect
605 dielectric screening: This prevents unfavourable interactions between charged and /or polar
606 residues within the protein molecule. This explains the residual biological function of the
607 enzyme. It appears therefore, that apart from water – striping effect of ethanol which
608 compromises the role of water as plasticiser, that ought to promote conformational flexibility,
609 the sucrose content may have rigidified the enzyme's three-dimensional structure. But there
610 is an apparent paradox considering the fact that sucrose is known as a folding stabilizer and
611 classified as an additive which shifts the folding equilibrium from the partially unfolded state
612 toward the native state [26]. It seems generally any plot versus folding destabiliser and
613 folding stabiliser should respectively give negative and positive *m*-value.

614 One may wish to add that, it is the enzyme primary structure that can determine the
615 effectiveness of a cosolute to unfold or rigidify its structure. Without residual biological
616 function of the enzyme, the determination of *m*-value based on kinetics/velocity of biological
617 function will be impossible. There is also the need to add that where there is negative *m*-
618 value there is preferential dehydration [3, 9]. There is a need also to suggest that the
619 presence of sucrose "unusually enhanced the effectiveness of ethanol to act as destabiliser"
620 (this is however, mere speculation) by rather, decreasing the solubility of the substrate. But
621 the plot versus sucrose, due perhaps to the concentration regime, exhibited in all except with
622 lowest concentration of ethanol, the usual positive *m*-values [Table 2b].

623 **Table 2b. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
624 **with the enzyme, in a reaction mixture, containing sucrose and ethanol-RTln$K_{eq(3)}$ as a**
625 **function of [Sucrose] (3.57, 7.19, 14.38, 28.76, 57.75mmol/).**
626

627 [Ethanol] 628 (mol/L)	~1.25	~3.22	~5.28
629 630 *m*-value 631 632 (kJL/mol^2)	– 27.93	28.55	276.69
633 634 r^2 635	0.87	0.53	0.96

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636 *The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [sucrose] with different concentration*
637 *of ethanol; (r^2) is the coefficient of determination.*
638
639 Unlike ethanol, aspirin showed what it may be, a folding destabiliser, having no
640 effect on substrate solubility which is unexpected considering the fact that while ethanol is a
641 solvent, aspirin is not. The *m*-values generated from the plot versus [Aspirin] with and
642 without sucrose yielded negative *m*-values (Table 3a). It thus, appears that aspirin is a
643 folding destabiliser to the enzyme, porcine pancreatic alpha-amylase. Therefore, as
644 explained by **Singh et al [27],** the critical factor is the partitioning between water and
645 osmolyte (in this case aspirin) at solvent-exposed surfaces of a protein whereby denaturing
646 cosolute accumulate or bind at the surface and promote unfolding as applicable to the effect
647 of aspirin on the enzyme.
648
649

650 **Table 3a. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
651 **with the enzyme, in a reaction mixture, containing sucrose and aspirin-RTIn$K_{eq(3)}$ as a**
652 **function of [Aspirin] (0.76, 3.05, 6.10 mmol/L).**
653

654 [Sucrose] 655 (mol/L)	0.00	7.19	14.38	28.76	57.75
656 *m*-value 657 (kJL/mol^2)	– 188.55	– 3754.56	– 4177.46	28.76	– 2174.34
658 659 r^2	0.87	1.00^z	1.00^z	0.99	0.99

660
661
662 *The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [Aspirin] with different concentration of*
663 *sucrose; the superscript z indicates datum from a straight line of two-data points; (r^2) is the*
664 *coefficient of determination.*
665
666 With a mixture of aspirin and sucrose the *m*-values from the plot versus [Sucrose]
667 were all positive (Table 3b) in line with the view that stabilizing osmolytes have an
668 overwhelming tendency to be excluded from the protein surface, forcing the polypeptide to
669 adopt a compactly folded structure with a minimum of exposed surface area. On this issue of
670 *m*-values, it is pertinent to note that it may not be unusual that sucrose was unable to totally

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671 refold rather than over-rigidify because it has been observed that similar observation was
672 made in respect of chymotrypsin, chymotrypsin, and ribonuclease [28].

673 **Table 3b. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
674 **with the enzyme, in a reaction mixture, containing sucrose and aspirin-RTIn$K_{eq(3)}$ as a**
675 **function of [Sucrose](3.57, 7.19, 14.38, 28.76, 57.75 mmol/L).**
676

677 [Aspirin] 678 (mol/L)	0.76	3.05	6.10
679 *m*-value 680 681 (kJL/mol^2)	41.10	96.39	57.45
682 683 r^2	0.74	0.80	1.00^z

684
685
686 *The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [sucrose] with different concentration*
687 *of aspirin; the superscript z indicates datum from a straight line of two-data points; (r^2) is the*
688 *coefficient of determination.*
689
690 Next is the issue of preferential solvation, hydration and osmolation, which has been
691 described as a thermodynamic quantity that describes the protein occupancy by the
692 cosolvent/water molecules [23]. The results in this research are based on either Eq. (29b) or
693 Eq. (34c) which shows direct link between the *m*-value and change in preferential interaction
694 parameter (PIP). With ethanol alone, the PIP values were unexpectedly negative (Table 4a).

695 **Table 4a. Preferential interaction parameters in a reaction mixture containing ethanol**
696 **as the only cosolute.**
697

698 [Ethanol] 699 (mol/L)	1.25	~2.4	~3.23	~4.31	~5.28
700 $\Delta_N^D\Gamma_{23}$	– 0.78	–1.49	–2.01	– 2.68	– 3.28

701 *The symbol $\Delta_N^D\Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition.*

702
703 This has been observed for chymotrypsin elsewhere [23]; but with the presence of sucrose
704 as part of ternary mixture of cosolutes, PIP values as a function of [Ethanol], showed positive
705 sign (Table 4b) because, *ab initio* the *m*-values were negative in sign. This is as expected if
706 the known effect of ethanol is taking into account. Such effect includes the change in the

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protein-water interactions and consequently, the modulation of the protein stability. The stripping of weakly bound water [9, 29] due to the binding of ethanol is inevitable, thereby leading to altered function of the enzyme. However, the PIP values as a function of [Sucrose], gave in all, except with lowest [Ethanol], negative values of PIP (Table 4c). This may be as a result of the greater solubilising effect of a higher concentration of ethanol on the insoluble raw starch.

Table 4b. Preferential interaction parameters in a reaction mixture containing ethanol and sucrose- $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$ is taken as a function of ethanol concentration.

	[Sucrose]/mmol/L				
	3.57	7.19	14.38	28.76	57.75
	$\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$				
[Ethanol] mol/L)					
~1.25	~0.86	–1.46	0.81	0.33	0.2
~3.23	~2.22	3.79	2.09	0.86	0.55
~5.28	3.64	6.20	3.42	1.40	0.90

The symbol $\Delta_N^D\Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it is obtained as a function of ethanol concentration (See either Eq. (34b) or Eq. (28b)) with different concentration of sucrose.

Table 4c. Preferential interaction parameters in a reaction mixture containing ethanol and sucrose - $\Delta_N^D\Gamma_{23}$ is taken as a function of sucrose concentration.

	[Ethanol]/mmol/L		
	~1.25	~3.23	~5.28
	$\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$		

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741 [Sucrose]/mmol/L

742 3.57	0.04	– 0.04	–3.83
743 7.19	0.08	– 0.08	–0.77
744 14.38	0.16	– 0.16	–1.54
745 28.76	0.31	– 0.32	–3.09
746 57.75	0.63	– 0.64	–6.20

747 *The symbol $\Delta_N^D\Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it*
748 *is obtained as a function of sucrose concentration (See either Eq. (34b) or Eq. (28b)) with*
749 *different concentration of ethanol.*
750

751 The PIP values as a function of [Aspirin] only conformed to conventional expectation
752 of being positive thereby suggesting a binding interaction with enzyme. The magnitudes
753 showed increasing trend (Table 5a). Also, the PIP values as a function of [Aspirin] with
754 different [Sucrose] were positive pointing to the fact that aspirin has a strong affinity for the
755 enzyme despite the presence of sucrose (Table 5b).

756 **Table 5a. Preferential interaction parameters in a reaction mixture containing aspirin**
757 **as the only cosolute.**
758

759 [Aspirin] 760 (mmol/L)	1.247	~2.398	~3.228	4.311	5.279
761 $\Delta_N^D\Gamma_{23}$	0.556	~2.398	~3.228	4.311	5.279

762 The symbol $\Delta_N^D\Gamma_{23}$ *is the preferential interaction parameter for folding-unfolding transition*.
763

764 **Table 5b. Preferential interaction parameters in a reaction mixture containing aspirin**
765 **and sucrose- $\Delta_N^D\Gamma_{23}$ is taken as a function of aspirin concentration.**
766

767	[Sucrose]/mmol/L			
768	7.19	14.38	28.76	57.75
769	$\Delta_N^D\Gamma_{23}$			
770 [Aspirin]/mmol/L				
771 0.76	1.107	1.231	0.723	0.641
772 3.05	4.441	4.941	2.902	2.572

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773 6.10 8.882 9.882 5.804 5.143

774 *The symbol* $\Delta_N^D\Gamma_{23}$ *is the preferential interaction parameter for folding-unfolding transition; it*
775 *is obtained as a function of aspirin concentration with different concentration of sucrose.*
776
777 The PIP values as a function of [Sucrose] with different [Aspirin] were negative
778 (Table 5c); this again conforms to the conventional behaviour of sucrose as a stabilising
779 osmolyte. This seems to suggest that the concentration regime of sucrose is sufficient to
780 cancel the initial effect of aspirin if the enzyme was incubated in an aqueous solution of
781 aspirin. Meanwhile, there are theories of preferential interaction which are Kirkwood-Buff,
782 cavity formation, solvophobic/solvophilic, surface tension theories *etc* with which to elucidate
783 the results. By being excluded from the peptide back bone as to imply solvophobic effect,
784 sucrose unlike ethanol and aspirin, is able to force protein to fold, leaving, as a
785 consequence, excess of the cosolute in the bulk solution. Here, according to Rösgen *et al* [3]
786 the Kirkwood-Buff theory comes into relevance. Thus an enrichment or relative excess of
787 water around protein corresponds to a positive G_{21} (positive correlation resulting from
788 exclusion), whereas a depletion of water around protein corresponds to a negative G_{21}
789 (negative correlation which is due to preferential binding) [7].

790 There is a recent theory implicating Lifshitz's dispersion forces which are inextricably
791 involved in solute-induced stabilization/destabilization of globular proteins [11]. The positive
792 and/or negative electrodynamic pressure generated by the solute–protein interaction
793 (perhaps as implied in Lifshitz's dispersion forces) across the water medium seems to be the
794 fundamental mechanism by which solutes affect protein stability [8] as against preferential
795 hydration or exclusion of cosolute.

796 As stated elsewhere [18] another aspect of the effect of sucrose is the energy cost
797 of cavity formation in order to accommodate the expanded conformation of the unfolded
798 enzymes. The free energy needed to accommodate the expanded form in the presence of
799 sucrose is high. Therefore, in line with Lech atelier principle, there was a shift towards the
800 direction of less expanded or more compacted species within native state ensemble [28, 30].
801 This may be as a result of exclusion of sucrose from the enzyme due to increase in surface

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802 tension of water occasioned by sucrose in a manner dependent on the proteins' surface area
803 [28, 30]. The increase in surface tension may explain the increase in the free energy cost for
804 cavity formation for the accommodation of the unfolded protein [28, 30]. If the case of
805 glycerol is a general one [31] then sucrose, may have achieved partial refolding of the
806 enzyme by strengthening hydrophobic interaction and by overcoming the unfavourable
807 electrostatic interaction between charged residues [31]. Since destabilisers and stabilisers
808 have opposing effects, one may conjecture that unlike sucrose, ethanol and aspirin which
809 bind may be decreasing the surface tension, reducing the energy cost for cavity formation for
810 the accommodation of the expanded unfolded enzyme.

811 **Table 5c. Preferential interaction parameters in a reaction mixture containing aspirin**
812 **and sucrose- $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$ is taken as a function of sucrose concentration.**
813
814
815
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826

	[Aspirin]/mmol/L		
	~1.25	~3.23	~5.28
		$\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$	
[Sucrose] mmol/L)			
7.19	– 0.115	– 0.269	– 0.160
14.38	– 0.229	– 0.537	– 0.320
28.76	– 0.458	– 1.075	– 0.641
57.75	– 0.921	– 2.159	– 1.287

827 *The symbol $\Delta_N^D\Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it*
828 *is obtained as a function of sucrose concentration with different concentration of aspirin.*
829
830 In summary, it is pertinent to state that lack of details occasioned by what may have
831 been considered as basic principles requiring less attention results in perceived technical or
832 conceptual error in well-intended research papers in literature. Although a dimensionally
833 consistent equation may be the case, it does not necessarily imply that the equation/model is
834 suitable for the qualitative and quantitative analysis of issues being addressed. On the other
835 hand the issue/concept being addressed may be clear, the theoretical background, both
836 qualitative and mathematical may become invalid if in particular, the mathematical models,
837 give results that are dimensionally inconsistent with the parameters to be determined. This is

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838 the hallmark of various observations in literature that motivated this research. The
839 contentious issue was precipitated by the observation in Eq. (19a), as found in literature,
840 which shows that the left hand side is dimensionless while the right hand side is not (unit is
841 litre/mol.). The appearance of $\left(\frac{\partial \mu_3}{\partial \text{In} C_3}\right)_{\text{T,P}}$ and $\frac{\partial \mu_3}{\partial C_3}$ in some equations in literature is one such
842 evidence of inconsistence making the value of hydrated molar volume of cosolute
843 contentious. Both parameters can be dimensionless if the mole fractions were to be the case
844 otherwise, some of the equations where they appear, become invalid. For instance Eq. (21)
845 and Eq. (23) are dimensionally inaccurate.

846 Combining Timasheff equation (Eq. (2)) with derived equation (Eq. (27a)) results in a
847 different slope and consequently the value of $G_{21} - G_{23}$which appeared as a reciprocal
848 equal to the slope is also different from what is expected from Eq. (28a). Also, the
849 introduction of apparent molar volume, V_1 into Eq. (41) for instance creates, *ab initio*, a
850 dimensionally consistent equation, including the derived equation for the determination of V_1.
851 The introduction of RT Ina_3 into Eq. (39) and V_1 into Eq. (41) gives after integration
852 equations which are dimensionally valid but not necessarily suitable equations for the
853 determination of V_1. Taking 1-1/γ_3 as a function of C_3 gives a better correlation, where V_1 is
854 a slope. The equation of unfolding has also been revisited, and deriving in the process,
855 alternative equations that are suitable for different situations in which velocity of amylolysis
856 as observed is either greater or less than the velocity for native untreated enzyme, with a
857 caveat that the observed velocity of hydrolysis for the treated enzyme is greater than for the
858 unfolded enzyme. The concept of preferential interaction and *m*-value were investigated by
859 treating the enzyme with three cosolutes, ethanol, aspirin, and sucrose.

860 This summary is imperatively terminated with following comment. The fact that
861 ethanol has been implicated in the aetiology of distinct intermediate protein states
862 responsible for numerous neurodegenerative diseases such as Alzheimer's disease,
863 Parkinson's disease, and Huntington's disease [23] should motivate the need for appropriate

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models that can be used to quantify the physico-chemical and biophysical effect of ethanol so as to establish a standard. This does not rule out improvisation as was the case in the thesis that generated the data; but the truth needs to be told as to the degree of precision of instrumentation. Stating otherwise to gain acceptance or evade censorship render quantitative result invalid and below standard in the light of the wishes of Strenda and what is expected of high precision instrumentation.

CONCLUSION

A major theoretical investigation was carried out on the issue of solution structure with a conclusion that it is as usual determined by either a relative excess or a deficit of the solution component either in the bulk or around the macromolecular surface domain; the preferential interaction coefficient or parameter remains thermodynamically an extensive parameter. Some of the derived equations may remain dimensionally invalid if standard reference concentration/activity is not substituted into such equations. All derived equations based on speculation or assumption except the equation derived from first principle may be useful for the determination of (G_{13} − G_{33}), the apparent hydrated molar volume of the osmolyte/cosolute. As with ethanol unlike aspirin, the *m*-values exhibit positivity contrary to the usual; the cognate preferential interaction coefficient has sign other than the usual with ethanol unlike with aspirin alone and with sucrose. In the light of earlier comment, it is hereby recommended that for feature research, scholars or researchers should against the backdrop of the theoretical exposition in this research carry out experiment with a-state-of-the-act high precision instrumentation so as generate very high quality data.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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895 **COMPETING INTERESTS**

896 There is no competing interest..

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979 **SUPPLEMENTARY TEXT**

980 **1. Apparent hydrated molar volume based on alternate equations to Eq. (19a)**

981 At this juncture, an alternative first view is to be given to Eq. (19a). In the first place,
982 there was no indication as to whether or not Eq. (19a) was the original contribution of the
983 authors [3]. Besides, no reference was made to literature. The side to be taken is that the
984 equation is the original contribution of the authors. The issue of dimensional inconsistency
985 has been established and it is very apparent. The equation is restated as

986 $$\frac{1}{1-C_3(G_{13}-G_{33})}=\left(\frac{\partial\Delta\mu_3}{R\partial T}\right)_{\mathrm{P}} \tag{38}$$

987 Equation (38) expresses accurate dimension because C_3V_1 is dimensionless. The value
988 of V_1, in this case is taken at a fixed concentration of the osmolyte at varying temperature
989 which expectedly affects the chemical potential of the osmolyte at constant pressure. The
990 concentration of the osmolyte can also be affected because, the density of the aqueous
991 solvent changes with temperature. But at a fixed thermodynamic temperature and pressure,

992 $$\frac{1}{1-C_3(G_{13}-G_{33})}=\left(\frac{\Delta\mu_3}{RT}\right)_{\mathrm{P,T}} \tag{39}$$

993 There is a deduction from Eq. (39) which is the issue of generalisation to both ideal and
994 nonideal solution of the osmolyte. The variable $\left(\frac{\Delta\mu_3}{RT}\right)_{\mathrm{P,T}}$ may be equal to InC_3 or Ina_3, if ideal
995 or nonideal case is applicable. However, in line with Levine [22], it seems more appropriate
996 to use In X_3 (for the idea case solution) and In γ_3X_3 (for the nonideal case). Nonetheless,

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997 rearrangement of Eq. (39) gives equation which shows clearly again that V_1 can only be seen

998 as constant quantity if obtained as a slope. The equation is

999
$$\frac{1-\text{In}a_3}{\text{In}a_3} = -\ C_3V_1 \qquad \text{(40a)}$$

1000
$$1 - \frac{1}{\text{In}a_3} = \ C_3V_1 \qquad \text{(40b)}$$

1001 However, if calculation is carried out, the value that is obtainable from Eq. (40a) and from the

1002 slope, if a plot is carried out, is much larger and positive compared to values that may be

1003 obtained from Eq. (31), Eq. (35b), and Eq. (37b).

1004 An alternative 2nd view is hereby given to Eq. (19a). For the core chemical physicist

1005 to proof is the introduction of apparent hydrated molar volume into Eq. (19a) to give

1006
$$\frac{V_1}{1-\ C_3V_1} = \frac{1}{RT}\left(\frac{\partial\mu_3}{\partial C_3}\right)_{\text{T,P}} \qquad \text{(41)}$$

1007 Since $\frac{1}{RT}\left(\frac{\partial\mu_3}{C_3}\right)_{\text{T,P}} = \partial\text{In}a_3/\partial C_3 \neq \frac{1}{1-\ C_3V_1}$, then what may be postulated is

1008
$$(\partial\text{In}a_3/\partial C_3)_{\text{T,P}} = \frac{V_1}{1-\ C_3V_1} \qquad \text{(42)}$$

1009 Next, one integrates as follows:

1010
$$\int \partial\text{In}a_3 = \int \frac{V_1}{1-\ C_3V_1}\partial C_3 \qquad \text{(43a)}$$

1011
$$\text{In}a_3 = -\text{In}(1-\ C_3V_1) \qquad \text{(43b)}$$

1012
$$1 - \frac{1}{a_3} = C_3V_1 \qquad \text{(44)}$$

1013 Nonetheless Eq. (44) remains conjectural until firmly proven by the core chemical physicist.

1014 The slope of the plot of left-hand side versus right-hand side will always yield a positive

1015 slope. The implication is that the apparent hydrated molar volume yielded from such plot can

1016 be exceptionally large for very dilute solution of the cosolute given that for ideal solution $a_3 \cong$

1017 C_3 unlike nonideal solution. The same is applicable, but to a greater extent, if mole fraction

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1018 is taken in place of a_3. Having used C_3 directly and InC_3 where applicable and having seen a

1019 clear dimensional inconsistency, there is need to consider the use of mole fraction of

1020 solution component as in literature [22]. Doing so is very likely to give very large slope as the

1021 apparent hydrated molar volume for the dilute solutions well above the values obtained using

1022 a_3 and γ_3.

1023 The place of standard reference molar concentration or activity has general

1024 implication. It is necessary to note that $\Delta\mu_3 = RT\text{In}C_\text{i} \neq RT\text{In}x_\text{i}$ where, x_i is the mole

1025 fraction of the solution component given as $n_\text{i}/(n_1 + n_\text{i})$ where respectively, n_i and n_1 are

1026 the number of moles of any solution components and water (usually ≅ 55.5556). For reason

1027 stated elsewhere [15], the equation, $\Delta\Gamma_{2\text{i}} = \frac{\text{In}K_\text{eq}}{\text{In}a_\text{i}}$ may need to be rewritten as $\Delta\Gamma_{2\text{i}} = \frac{\text{In}K_\text{eq}}{\text{In}a_\text{i}/a_\text{i}^0}$

1028 which is never equal to $\Delta\Gamma_{2\text{i}} = \frac{\text{In}K_\text{eq}}{\text{In}(\gamma_\text{i}x_\text{i})}$.

1029 **2. The reexamination and derivation of the model equations for the**

1030 **determination of the equilibrium constant, for the transition from folded to unfolded**

1031 **protein.**

1032 The reexamination and derivation of equations for the determination of equilibrium

1033 constant for the transition from folded to unfolded protein is the purpose of this subsection.

1034 Meanwhile, there is the need to make further modification of Baskakov and Bolen equation

1035 [32]. The equation seems to suggest that the equilibrium constant for folding-unfolding

1036 transition may be increasing with increasing concentration of the protecting osmolyte in

1037 particular in the presence of a known destabilizing cosolute. This is against the backdrop of

1038 the fact that the specific activity of the enzyme may be increasing with increasing

1039 concentration of the protecting osmolyte. The paradox is that $[U]/[F] > 1$ for such a case.

1040 Here, *U* and *F* are respectively the unfolded and folded protein. The conformational

1041 adjustment by partial unfolding does not amount to instability. The issue of conformational

1042 flexibility for function dictated by the environment is well studied [33, 34]. The 2nd paradox is

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1043 that the *m*-value should also be negative even if the specific activity of the enzyme is > the
1044 native activity.

1045 Another aspect is that the specific activities though > native activity are nevertheless
1046 decreasing with increasing concentration of the protecting osmolyte. In this case the values
1047 of the equilibrium constant would be decreasing against what is expected from the general
1048 relation $K_{eq} = [U]/[N]$. The implication is that the *m*-value would be positive. While such
1049 positive sign may agree with the definition of *m*-value for a protecting osmolyte it will not
1050 correlate with the result from the plot of ln $(1/K_{\mathrm{eq}})$ versus $[C_{\mathrm{os}}]$ if increasing specific activities
1051 may be observed with increasing concentration of the protecting osmolyte and if the original
1052 equation given below is used for the calculation of K_{eq} after substituting velocity data into it.

1053 $$K_{\mathrm{eq}} = \frac{V_{\mathrm{Max}} - V_{\mathrm{N}}}{V_{\mathrm{N}} - V_{\mathrm{Min}}} = [U]/[N] \quad (45)$$

1054 Increasing magnitude of V_{Max}, a function of $[N]$, appears to suggest that the magnitude of $[U]$
1055 is increasing which may be incorrect. Therefore, if V_{Max} is increasing with increasing $[C_3]$, an
1056 alternative equation is needed so that calculated values of K_{eq} with increasing $[C_3]$ (for
1057 protecting osmolyte in particular) should be decreasing because in such a case, $[U]$ may be
1058 decreasing; this should be expected from calculations using such an equation that
1059 corresponds to $K_{\mathrm{eq}} = [U]/[N]$ if the indicator of folded or refolding enzyme, the velocity of
1060 catalytic action, is increasing.

1061 Another scenario is the specific activity which may be > unfolded enzyme specific
1062 activity but < the native enzyme activity even with increasing concentration of the protecting
1063 osmolyte in the presence of strong destabiliser. The specific activities may also be
1064 increasing but < native activity. In this case, the original Baskakov and Bolen equation [32]
1065 cannot apply. While the equations that are to be derived shortly may not be sacrosanct, the
1066 preceding issues cannot easily be ignored.

1067 The equilibrium constant (K_{eq}) for the process folded (*F*)→unfolded (*U*) is adapted
1068 from Pace equation [35] and modified Baskakov and Bolen equation [32] as in previous

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1069 publications [15-16, 18]. First is the equation for the assay in which the catalytic velocity of
1070 the enzyme is increasing with increasing concentration of the osmolyte. Such velocities may
1071 be < velocity of the native enzyme in a reaction mixture containing destabilising cosolute and
1072 increasing concentration of the protecting osmolyte. There may be increasing velocities with
1073 increasing concentration of the destabilising osmolyte only, but such velocities may also be
1074 < velocity of the native enzyme. This is to say that the observed velocities are < the velocity
1075 of the native enzyme which is either in a binary mixture or a ternary mixture of osmolyte.
1076 Pace defines mathematically [*U*] (this is however a fraction of the protein that is unfolded, the
1077 symbol [*U*] notwithstanding; this applicable to [*N*] for the folded) as

1078 $$\frac{A_{\mathrm{N}}-A_{\mathrm{OBS}}}{A_{\mathrm{N}}-A_{\mathrm{MIN}}}=[U] \qquad (46)$$

1079 Where A_{N}, A_{OBS}, and A_{MIN} are absorbance of the native enzyme, the observed absorbance
1080 used to follow unfolding in the transition region, and the absorbance of the unfolded protein
1081 respectively. In place of the absorbance of the protein the absorbance of the product (within
1082 the visible region of the spectrophotometer) is taken and converted to the molar
1083 concentration of the product. Equation (46) takes the form for the case just described as
1084 follows.

1085 $$\frac{V_{\mathrm{N}}-V_{\mathrm{OBS}}}{V_{\mathrm{N}}-V_{\mathrm{MIN}}}=[U] \qquad (47)$$

1086 Therefore,

1087 $$[N]=1-[U] \qquad (48a)$$

1088 Substitution of Eq. (47) into Eq. (48a) gives after rearrangement

1089 $$[N]=\frac{V_{\mathrm{OBS}}-V_{\mathrm{MIN}}}{V_{\mathrm{N}}-V_{\mathrm{OBS}}} \qquad (48b)$$

1090 Therefore, Eq. (46) takes the modified form after replacing [*U*] and [*N*] with Eq. (47) and Eq.
1091 (48b) respectively to give

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1092 $$K_{\mathrm{eq}} = \frac{V_{\mathrm{N}} - V_{\mathrm{OBS}}}{V_{\mathrm{OBS}} - V_{\mathrm{MIN}}} \quad (49)$$

1093 Here, in Eq. (49), $V_{\mathrm{N}} > V_{\mathrm{OBS}} > V_{\mathrm{MIN}}$.

1094 Next is the equation for the assay in which the catalytic velocity of the enzyme is
1095 increasing with increasing concentration of the protecting osmolyte. Such velocities may be
1096 > velocity of the native enzyme in a reaction mixture containing destabilising cosolute and
1097 increasing concentration of the protecting osmolyte. The equation is also relevant to the
1098 case in which the velocities are increasing for the treated enzyme with increasing
1099 concentration of the osmolyte. Such velocities should also be > the velocity of the untreated
1100 native enzyme. To begin with it is imperative to realise that the original equation by Pace [35]
1101 concerns the unfolding enzyme. It can be adapted for the refolding case leading to
1102 hydrodynamic radius equal to or less than the radius of the native enzyme (if there is extra-
1103 rigidification that is not very common).

1104 $$[N] = \frac{A_{\mathrm{MAX}} - A_{\mathrm{N}}}{A_{\mathrm{MAX}} - A_{\mathrm{MIN}}} \quad (50)$$

1105 Where, the parameter A_{MAX} is the absorbance of the refolded or over-folded protein. Then
1106 replacing the parameters with the velocity of catalytic action of the protein gives

1107 $$[N] = \frac{V_{\mathrm{MAX}} - V_{\mathrm{N}}}{V_{\mathrm{MAX}} - V_{\mathrm{MIN}}} \quad (51)$$

1108 Thus substitution of Eq. (51) into $[U] = 1 - [N]$ gives

1109 $$[U] = \frac{V_{\mathrm{N}} - V_{\mathrm{MIN}}}{V_{\mathrm{MAX}} - V_{\mathrm{MIN}}} \quad (52)$$

1110 Therefore, the equilibrium equation should be

1111 $$K_{\mathrm{eq}} = \frac{V_{\mathrm{N}} - V_{\mathrm{MIN}}}{V_{\mathrm{MAX}} - V_{\mathrm{N}}} \quad (53)$$

1112 In Eq. (53), $V_{\mathrm{MAX}} > V_{\mathrm{N}} > V_{\mathrm{MIN}}$. The nominator is constant for the system but the denominator
1113 is increasing with increasing values of V_{MAX}; this means that [*U*] may be decreasing such that
1114 K_{eq} or [*U*]/[*N*] is decreasing as expected for a refolding protein.

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