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1 **Controlling protein stability: mechanisms revealed using formulations of arginine, glycine and**
2 **guanidinium HCl with three globular proteins.**

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8 **Keywords:** calorimetry, DSC, aggregation, stabilization, formulation, osmolyte

9

10 **Abstract**

11 Three distinct interactions between the amino acid arginine and a protein explain arginine's ability
12 to modulate the thermal stability of proteins. Arginine's effect on the protein unfolding behaves like
13 the sum of its constituent parts, glycine and the guanidinium ion. The authors propose that glycine
14 can affect the thermal stability of a protein in two ways: (1) direct interaction with the charged side
15 chains and/or the peptide backbone of the protein which is observed at low concentrations and (2)
16 competition for water between the unfolding protein and the cosolute increasing the energy
17 required to hydrate the unfolding protein. The guanidinium ion acts by (3) direct interaction with
18 apolar regions exposed during unfolding reducing the energy required to hydrate the unfolding
19 protein.

20

21 **1. Introduction**

22 In recent years amino acids and other osmotically active organic molecules have found extensive
23 application as excipients in biopharmaceutical formulations (Arakawa et al., 2007; Bye et al., 2014).
24 This is due to their apparent positive effects on the solubility and stability of proteins; a consequence
25 of their ubiquitous nature in cells of almost all organisms. One such small molecule arginine is
26 commonly used as an additive in therapeutic protein manufacture and storage due to its reported
27 ability to solubilize proteins from inclusion bodies and prevent aggregation during chromatographic
28 purification steps. Its popularity as an additive stems from the fact that it appears to increase the
29 solubility of proteins without decreasing stability (Arakawa et al., 2007), unlike traditional
30 chaotropes such as urea and guanidinium which have negative effects on stability making them
31 unsuitable excipient candidates. However, the mechanism by which arginine achieves this seemingly
32 unique effect is still open to conjecture, and despite numerous attempts a consensus has not yet
33 been reached. If the mechanisms by which arginine acts can be comprehensively described and
34 understood, its effect on proteins can be predicted more accurately making its use as an excipient
35 more reliable.

36 The structural similarity of arginine's side chain to guanidinium has been linked to its ability to act
37 like a chaotropic salt in terms of solubilizing proteins and inhibiting aggregation (Xie et al., 2004).
38 However, differences in their effects on protein solubility and stability have prompted alternative
39 theories (Ishibashi et al., 2005). One point that has largely been ignored is the role other constituent
40 groups of the arginine molecule play in its action on protein stability, specifically the peptide end
41 which closely resembles a glycine molecule. Glycine is another commonly used amino acid in protein
42 formulations and a well-known stabilizer. Arginine's effect on structural stability of proteins appears
43 to vary depending on the protein studied, with no effect seen on the melting temperature of
44 lysozyme or RNase A (Arakawa et al., 2007; Reddy et al., 2005) but varying effects on that of
45 monoclonal antibodies (Thakkar et al., 2012), which has made it much harder to establish a
46 molecular mechanism for arginine's action. A lack of data reflecting the effect of lower
47 concentrations (<100 mM) of arginine on protein stability has also contributed to the absence of a

48 consensus. This is despite excipients regularly being used at these low concentrations, which are also
49 physiologically relevant. Hofmeister salts have recently been shown to affect protein stability via
50 different mechanisms at concentrations below 10 mM (Bye and Falconer 2013; Bye and Falconer
51 2014), which brings to question whether more complex small molecules follow a similar trend.
52 Furthermore, structural stability studies of proteins in the presence of additives are routinely carried
53 out in solutions with salt-containing buffers, which are also known to have an effect on protein
54 stability. These buffers could mask or enhance the effect of the additives by competitively binding to
55 charged amino acid side chains on the protein therefore blocking the binding of the arginine.

56 The aim of this study is to quantify the effects of arginine on the thermal stability of three well
57 characterized proteins, particularly at low concentrations (<100 mM) and in the absence of
58 potentially competing buffers and salts. The study will challenge the hypothesis that arginine
59 effectively acts as a guanidinium hydrochloride and a glycine linked by a three carbon chain linker. A
60 comparison of arginine effects to those of both guanidinium hydrochloride and glycine should bring
61 to light how the different structural components of the arginine molecule contribute to its actions
62 on protein stability.

63 **2. Materials and methods**

64 Bovine serum albumin (BSA), myoglobin from equine skeletal muscle & lysozyme from chicken egg
65 white, sodium octanoate and the cosolutes L-arginine, glycine and guanidinium hydrochloride
66 (GdnHCl) were all purchased from Sigma-Aldrich. The proteins were dissolved in and dialyzed against
67 HPLC grade water overnight at 4 °C using a Mini 8 kDa membrane dialysis kit (GE Healthcare, Little
68 Chalfont, UK) prior to experiments being carried out. 10 mM sodium octanoate was added to the
69 BSA solution as the protein was unstable in its absence, which should not have an effect on the
70 interaction with the cosolutes due to the specificity of its binding. Concentrations of 1 g BSA/l, 1 g
71 Lysozyme/l and 0.5 g Myoglobin/l were used at pH 7, and 8 respectively. These proteins were chosen
72 as they follow a two-state transition during unfolding, which is necessary to analyze the data and

73 they also represent a selection of differing net charges and sizes. Stocks of the cosolutes were made
74 to 2 M for glycine and guanidinium, and 1 M arginine, representing its solubility limit, and were also
75 adjusted to the appropriate pH.

76 Thermal stability of the proteins was established by Differential Scanning Calorimetry (DSC) using a
77 Nano-DSC (TA Instruments, New Castle, DE, USA) at a scan rate of 1.5 °C/min from 30 °C to 100 °C.
78 Software provided with the equipment was used to analyze the data, water-water baselines were
79 subtracted from the sample data and the temperature of maximum unfolding of the protein (T_m)
80 was calculated as the point at which the maximum relative heat capacity occurred. DSC scans of
81 each protein run repeatedly throughout the day show a precision in T_m of 0.23 °C for BSA, 0.41 °C for
82 myoglobin and 0.31 °C for lysozyme.

83 **3. Results and discussion**

84 The relative change in the T_m values of the proteins with the addition of increasing concentrations of
85 the cosolutes (arginine, glycine and guanidinium HCl) were calculated from DSC scans (Figure 1). At a
86 concentration of 1 M, glycine stabilized the proteins (Figure 2a) and guanidinium hydrochloride
87 destabilized at the same concentration (Figure 2b), as previously described (Bruździak et al., 2013;
88 Arakawa and Timasheff 1983) . At a concentration of 500 mM, arginine also appears to destabilize,
89 although not as strongly as guanidinium (Figure 2c), as previously described (Xie et al., 2004;
90 Ishibashi et al., 2005). For all three cosolutes their effects are more pronounced above 100 mM
91 (Figure 2) and these effects are similar for the three proteins, meaning the three cosolutes exert the
92 same effect regardless of protein charge and size. This is particularly apparent as lysozyme is
93 positively charged at pH 7, and both BSA and myoglobin are negatively charged at pH 7 & 8
94 respectively, yet there is no discernible difference in trend between the differently charged proteins
95 above 100 mM, and lysozyme sits between BSA and myoglobin for both arginine and glycine at
96 cosolute concentrations below 100 mM (Figure 2a&c). As a result electrostatic interactions can be
97 ruled out as a major mechanism for interaction in this case; as arginine and guanidinium

98 hydrochloride are positively charged molecules you would expect their effects to be more
99 pronounced for the oppositely charged BSA and myoglobin.

100 *3.1 Glycine*

101 Glycine stabilized all three proteins at glycine concentrations above 100 mM as previously reported
102 (Khan et al., 2013; Santoro et al., 1992). Below 100 mM more protein-specific effects are seen. At 50
103 mM glycine BSA T_m value was increased by 0.9 °C and lysozyme was relatively unaffected with a T_m
104 value just 0.4 °C above pure water. Myoglobin on the other hand was destabilized by the presence of
105 glycine with a reduction of the T_m value by 1.0 °C (Figure 2a). This has not been recorded in previous
106 publications. DSC scans of BSA in the presence of increasing concentrations of glycine demonstrate
107 that the change in heat capacity between the folded (pre-peak) and unfolded (post-peak) protein is
108 unaffected by the presence of glycine (Figure 1a). This suggests that direct interaction between the
109 glycine and the apolar interior of the protein exposed on unfolding is unlikely as you would expect to
110 see a difference in the change in heat capacity.

111 The T_m values for glycine at different concentrations suggest two phases with distinct mechanisms
112 that affect the thermal stability of proteins. A single mechanism such as preferential hydration
113 cannot explain the capability to destabilize then stabilize a protein as seen with myoglobin.

114 Kosmotropic anions have also been shown to have multiple concentration-dependent mechanisms
115 of action on thermal stability of lysozyme (Bye and Falconer 2014). These were attributed to
116 interaction with charged side chains at low concentrations, interaction with the peptide backbone at
117 intermediate concentrations and competition for water between the unfolding protein and the salt
118 at higher concentrations.

119 Glycine being a zwitterion has the ability to interact with the protein via both its negative and
120 positive charged groups, and due to its small size it is not sterically hindered from binding to multiple
121 parts of the protein. The number of potential sites for these weak interactions will vary depending

122 on the shape and charge of the protein, which results in the differences between glycine's effects on
123 the stability of the three proteins below 100 mM. In the case of glycine the destabilization observed
124 for the negatively charged myoglobin was not observed for the negatively charged BSA suggesting
125 that if there is an interaction with charged side chains it did not have a consistent effect on thermal
126 stability. The authors suggest that at concentrations below 100 mM the effect of glycine on protein
127 thermal stability could be attributed to interaction with the peptide backbone. It is well known that
128 interactions or repulsions with the peptide backbone play a major role in protein stabilization (Liu et
129 al., 1995; Street et al., 2006).

130 At concentrations above 100 mM glycine stabilizes all three proteins. This can be attributed to
131 competition for water between the unfolding protein and the cosolutes, similar to that seen for salts
132 (Bye and Falconer 2014). Both competition for water and preferential hydration (Timasheff 2002))
133 can be used to explain the increase in thermal stability in terms of an increase in energy required to
134 hydrate the protein as it unfolds. The competition for water theory suggests that the protein has to
135 compete with the cosolute for the water while preferential hydration suggests the phenomenon can
136 be expressed in terms of water molecules being preferentially bound to the protein due to the
137 presence of the cosolute in the bulk phase. The concentration-dependent stabilization seen here
138 corresponds to the competition for water theory. The authors suggest that below 100 mM glycine
139 there is still free bulk water present that can hydrate the newly exposed core of the protein upon
140 unfolding, which is why stabilization is unaffected by this mechanism at low concentrations. Once
141 the concentration of glycine in the bulk reaches 100 mM the protein has to compete for the water
142 that is 'locked up' in the hydration layer of glycine resulting in stabilization of the protein. The
143 'enhanced' structured water in the hydration shell of glycine has previously been seen using FTIR
144 and been used as an explanation for protein stabilization (Bruździak et al., 2013).

145 The protein-specific and largely unpredictable effect of glycine at concentrations below 100 mM is
146 particularly important as glycine is used in biopharmaceutical formulations and is naturally present

147 as an osmolyte in cells at these low concentrations. Destabilization by osmolytes has recently been
148 documented under a variety of conditions (Singh et al., 2011), and destabilization at low
149 concentrations followed by stabilization at higher concentrations has been seen with polyols and
150 glucose (Romero et al., 2009). The fact that this trend has not been observed before with amino
151 acids could simply be an artefact of the scarcity of data for protein stability in presence of osmolytes
152 at concentrations below 100 mM. There is also a tendency for researchers to use salt-containing
153 buffers in protein stability experiments, which could be screening these effects at lower
154 concentrations. Therefore this phenomenon may actually be true for many if not all osmolytes.

155 *3.2 Guanidinium Hydrochloride*

156 Guanidinium hydrochloride destabilized all three proteins by up to 17 °C between 100 mM and 1 M
157 as expected due to its well-known chaotropic action on proteins (Figure 2b). The change in T_m value
158 was not proportional to the denaturant concentration as predicted from previous work on the
159 apparent free energy of unfolding in the presence of guanidinium hydrochloride (Greene and Pace
160 1974). Deviation from a linear relationship was particularly pronounced for BSA and myoglobin.
161 Below 100 mM guanidinium hydrochloride has no notable effect on the melting temperature of any
162 of the three proteins meaning its denaturing effect only occurs at higher concentrations, which
163 makes its mechanistic action harder to explain. It is generally agreed upon that guanidinium binds to
164 the protein (Courtenay et al., 2001; Lee et al., 1974; Möglich et al., 2005). Most studies suggest that
165 this is via hydrogen bonding to the peptide backbone (Street et al., 2006; Robinson et al., 1965).
166 However, recent work by Lim et al. (2009) using measurement of acid and base catalyzed hydrogen
167 exchange by NMR has suggested that, although most denaturants act via this mechanism,
168 guanidinium hydrochloride does not. DSC scans of the unfolding of BSA in the presence of
169 guanidinium hydrochloride show different patterns of unfolding and an increase in the heat capacity
170 of the unfolded state compared to the folded (Figure 1b), which suggests that the guanidinium
171 cation is binding to the protein. Here we suggest that guanidinium acts like a detergent; the weakly

172 hydrated cation is pushed onto apolar parts of the protein due to strong water-water interactions, as
173 originally proposed by Collins (1995). This reduces the energy required to hydrate the apolar core of
174 the protein exposed during unfolding, resulting in a destabilization of the protein. As this is a weak
175 interaction governed mainly by water-water interactions, the destabilizing effect on the protein is
176 not seen until guanidinium hydrochloride is present at concentrations above 100 mM.

177 *3.3 Arginine*

178 Below 100 mM arginine acts very similarly to glycine in that protein-specific stabilization of BSA,
179 destabilization of myoglobin and neutral effects on lysozyme are seen. Above 100mM it begins to
180 destabilize all three proteins in a similar manner to the guanidinium, although not as strongly (Figure
181 2c). Arginine's mechanism of action is thought to be complex, as demonstrated by varying effects on
182 protein stability and concentration-dependent actions (Thakkar et al., 2012; Falconer et al., 2011).
183 However, current suggestions of weak transient interactions at low concentrations (Lim et al., 2009)
184 and preferential exclusion due to increase in surface tension and self-association or 'stacking' of
185 arginine molecules at higher concentrations (Shukla and Trout 2011; Das et al., 2007; Kita et al.,
186 1994; Vondrášek et al., 2009) cannot be used to explain the trends presented in this paper as this
187 would result in a stronger stabilization at higher concentrations. Instead we propose that arginine is
188 indeed acting as a mixture of its structural components and that the mechanisms for glycine and
189 guanidinium hydrochloride also explain arginine's functionality. The authors propose that the
190 confusion about arginine is due to the fact that it can affect the thermal stability of a protein in
191 multiple ways including the interaction with charged side chains and/or the peptide backbone,
192 competition for water (between the unfolding protein and the cosolute) and the interaction with
193 apolar regions exposed during unfolding (here referred to as a "wetting agent" effect). At
194 concentrations below 100 mM the overriding effect of arginine on protein stability is governed by its
195 glycine group, which is interacting weakly with the peptide backbone and exposed polar groups as
196 explained in section 3.1 causing a protein-specific effect. Above 100 mM the denaturing action of the

197 guanidinium group comes into effect as described in section 3.3, which involves the arginine
198 molecules being forced onto apolar regions of the protein, causing unfolding to be more favorable
199 due to it being energetically easier to hydrate the hydrophobic core of the protein. The strength of
200 the destabilization by arginine above 100 mM is weaker than that of the guanidinium alone, which
201 can be explained by the competing effects of the guanidinium and glycine portions of the arginine
202 molecule simultaneously competing for water, which increases the free energy of unfolding, and
203 having a wetting agent effect, which reduces the free energy of unfolding.

204 *3.4 Implications for formulation design*

205 The importance of using reliable excipients in biopharmaceutical formulations to stabilize
206 therapeutic proteins that would otherwise be unstable must not be underestimated. Both arginine
207 and glycine are regularly used as stabilizing excipients in biopharmaceutical formulations at
208 concentrations below 100 mM (Parkins et al., 2000). The data shown here demonstrates that both
209 small molecules can act in a more complex manner than first thought, with trends that exhibit
210 concentration-dependence and protein-specificity. Both of these characteristics could potentially
211 prevent arginine and glycine from being ideal excipient candidates as it is difficult to predict under
212 which specific conditions they exert the desired stabilizing effect. Based on the finding that arginine
213 acts like a combination of its constituent groups, the authors suggest using a mixture of glycine and
214 guanidinium hydrochloride in the place of arginine in formulations. This presents the advantage of
215 being able to specifically alter the ratios of the stabilizing glycine molecule and the solubilizing and
216 aggregation-preventing guanidinium molecule, in order to more accurately create the optimum
217 conditions for long-term storage of therapeutic proteins in aqueous solutions.

218 **4. Conclusions**

219 We have shown that arginine acts on protein stability like a combination of its constituent groups.
220 Below 100 mM arginine acts like glycine, above 100 mM it shows destabilizing effects similar to
221 guanidinium hydrochloride. The glycine alone demonstrates two stages of stabilization. The first

222 effect (at concentrations below 100 mM) is protein specific and is probably due to multiple direct
223 interactions with the polar or charged side chains and the partial charges on the peptide backbone
224 of the protein. The second stage (at concentrations above 100 mM) is similar to high charge density
225 anions where it was ascribed to competition for water between the unfolding (Bye and Falconer
226 2014). Guanidinium hydrochloride acts by direct interaction with apolar regions exposed as the
227 protein unfolds, which we call a “wetting agent” effect reducing the free energy required to hydrate
228 the protein’s interior as it unfolds.

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231 University of Sheffield for financing Lauren Platts’ studentship, and the Engineering and Physical
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306

307 **Figure Titles**

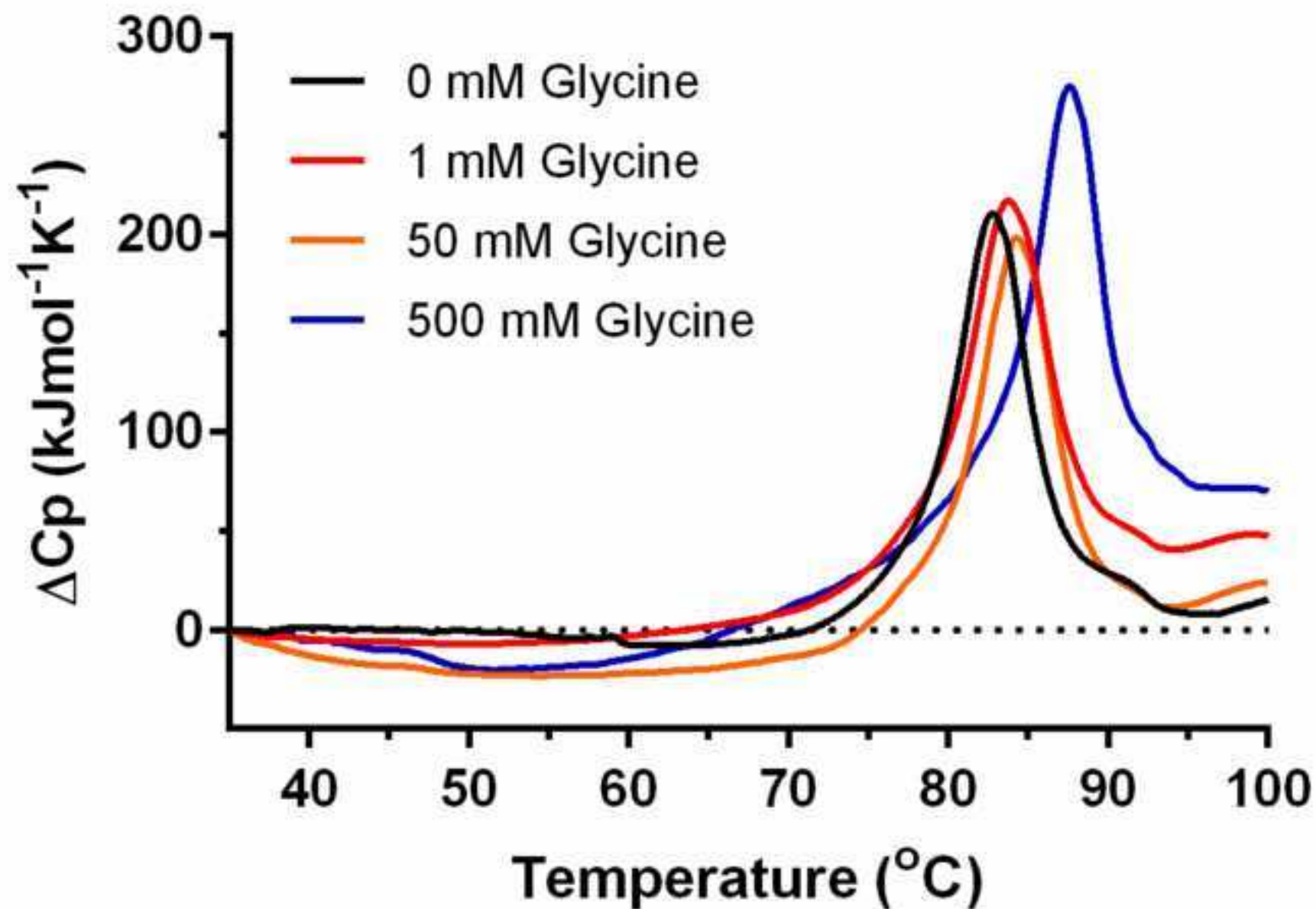
308

309 Figure 1. Representative DSC Scans of 1g/l BSA with 10mM sodium octanoate at pH 7 with different
310 concentrations of (a) glycine, (b) guanidinium hydrochloride and (c) arginine to show changes in T_m
311 represented by highest point of relative heat capacity and changes to unfolding pattern. BSA without
312 additives is plotted in each panel for comparison. A water baseline has been subtracted and initial
313 heat capacities have been normalized.

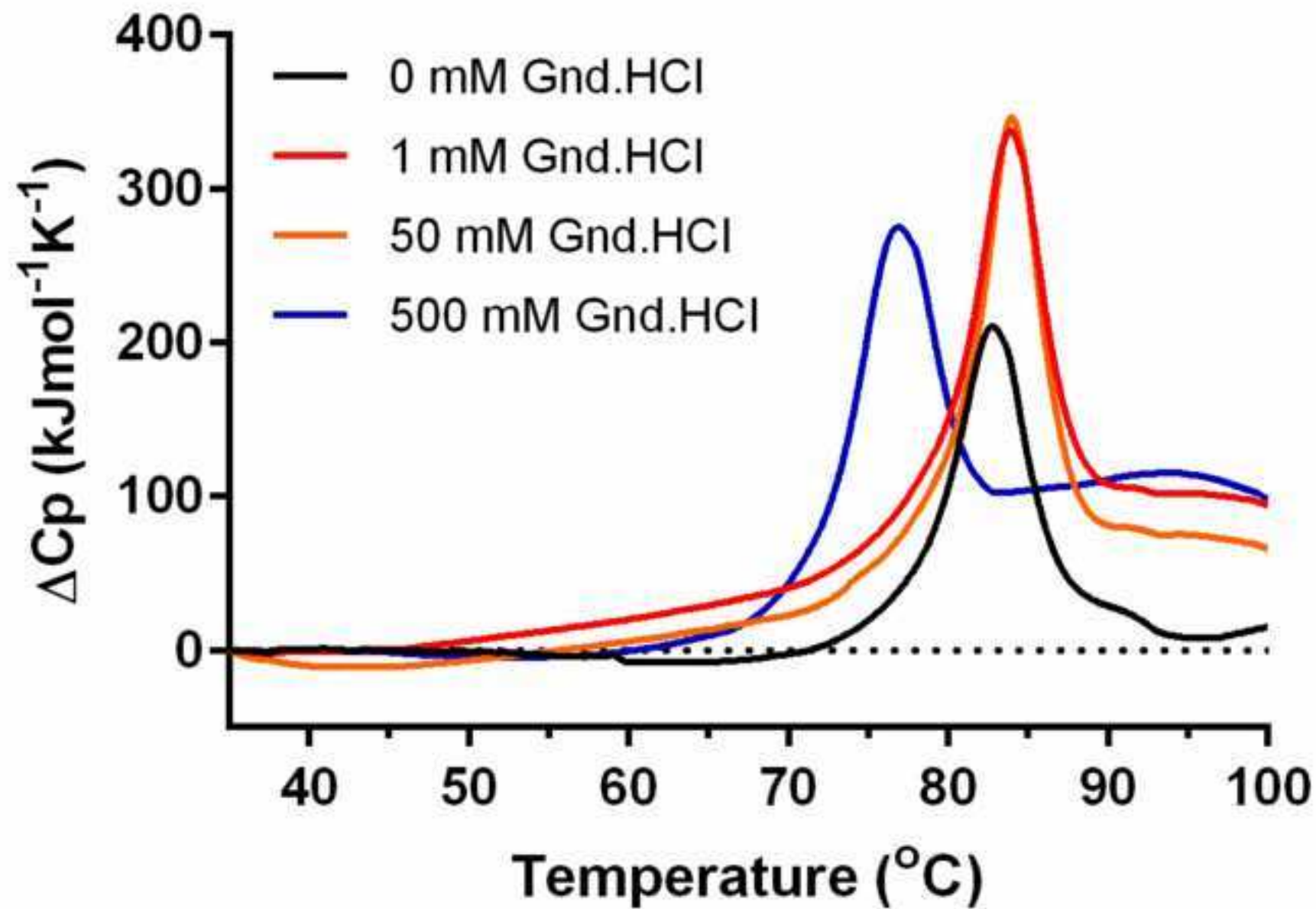
314 Figure 2. Relative change in the T_m of 1 g/L BSA at pH 7 (circles), 1 g/L lysozyme at pH 7 (triangles)
315 and 0.5 g/L myoglobin at pH 8 (squares) between the protein solution with no additives present and
316 in the presence of increasing concentrations of (a) glycine, (b) guanidinium hydrochloride and (c)

317 arginine plotted on a logarithmic scale. Dotted line shows no relative change in the T_m of the protein
318 to guide the eye.

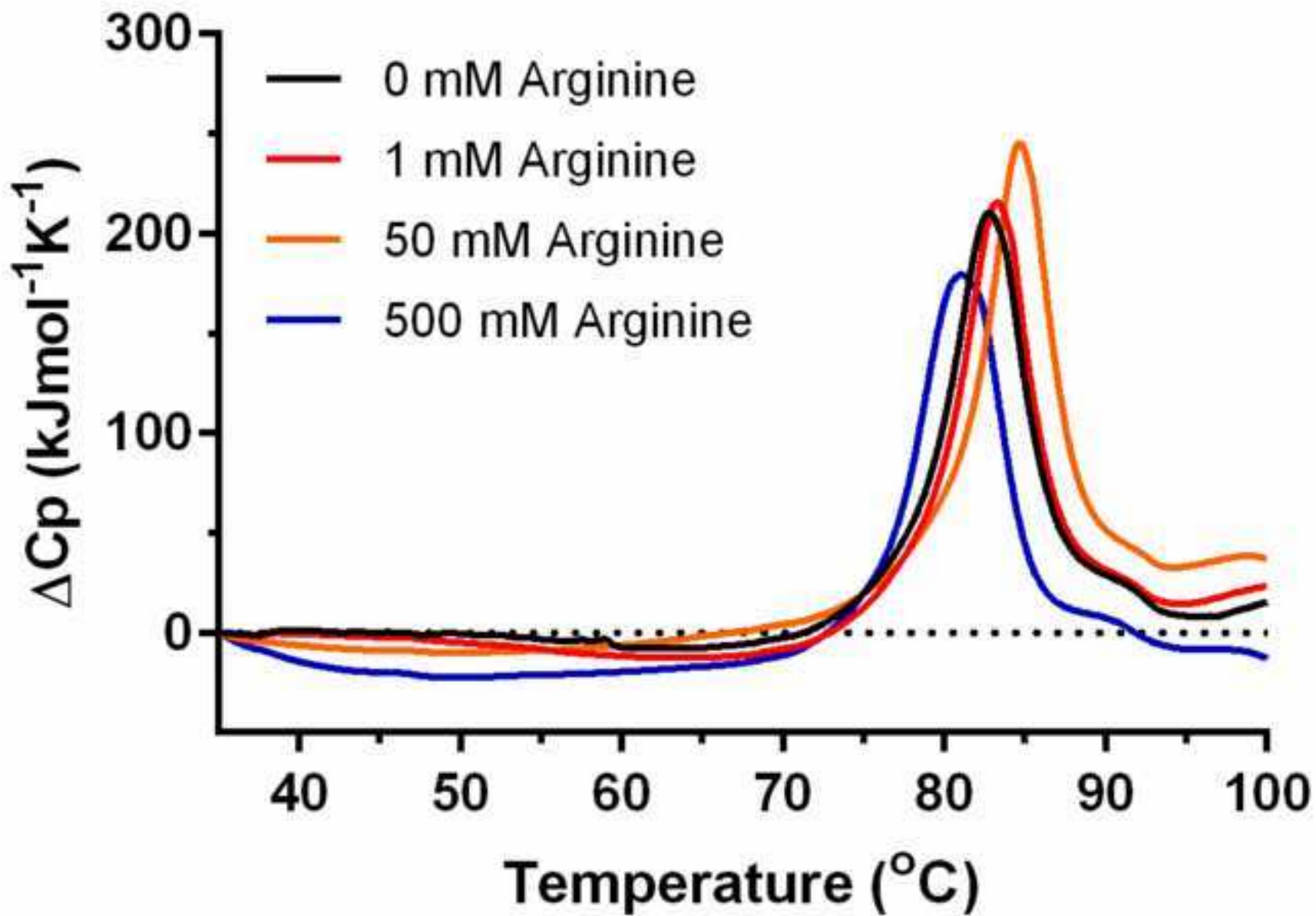
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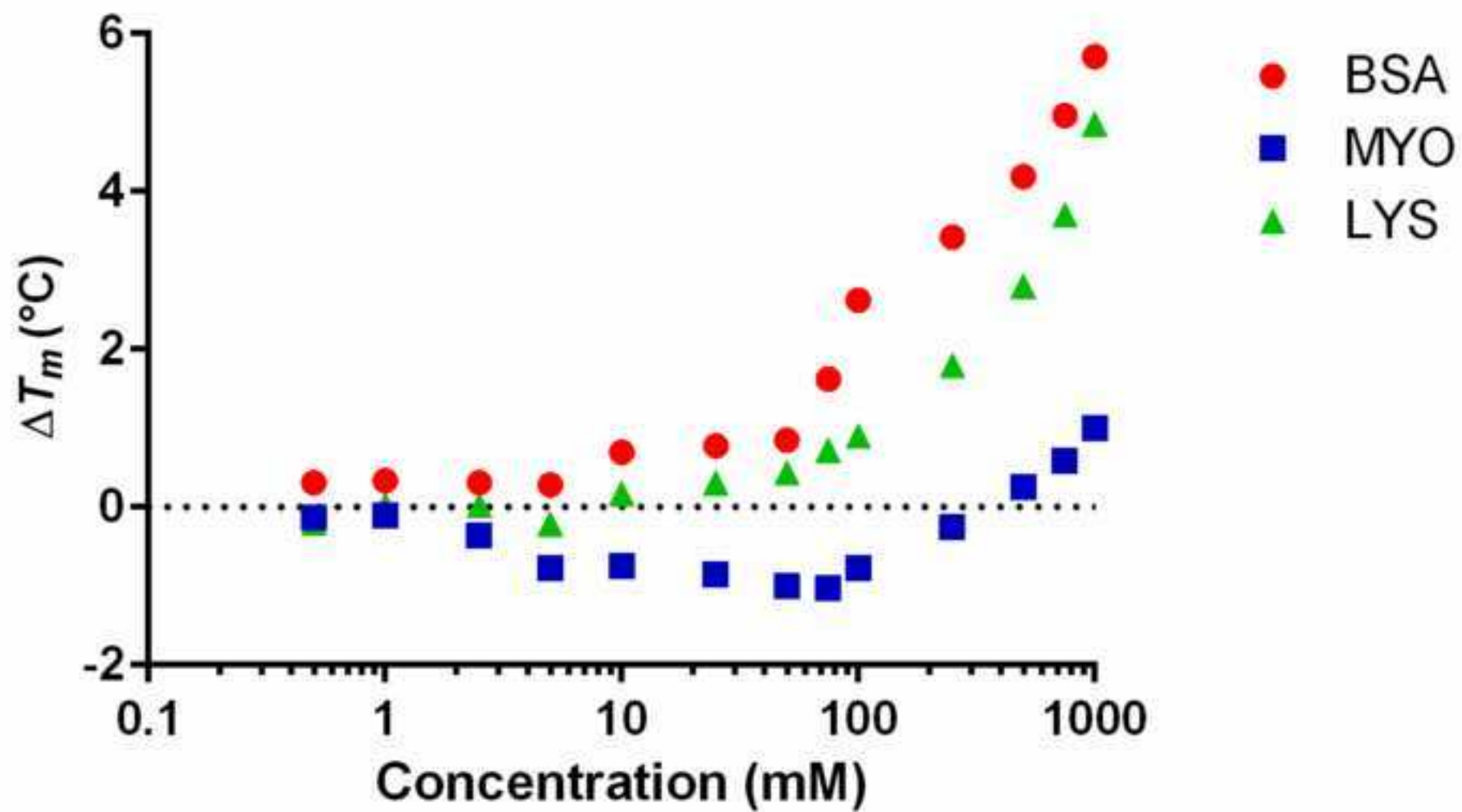
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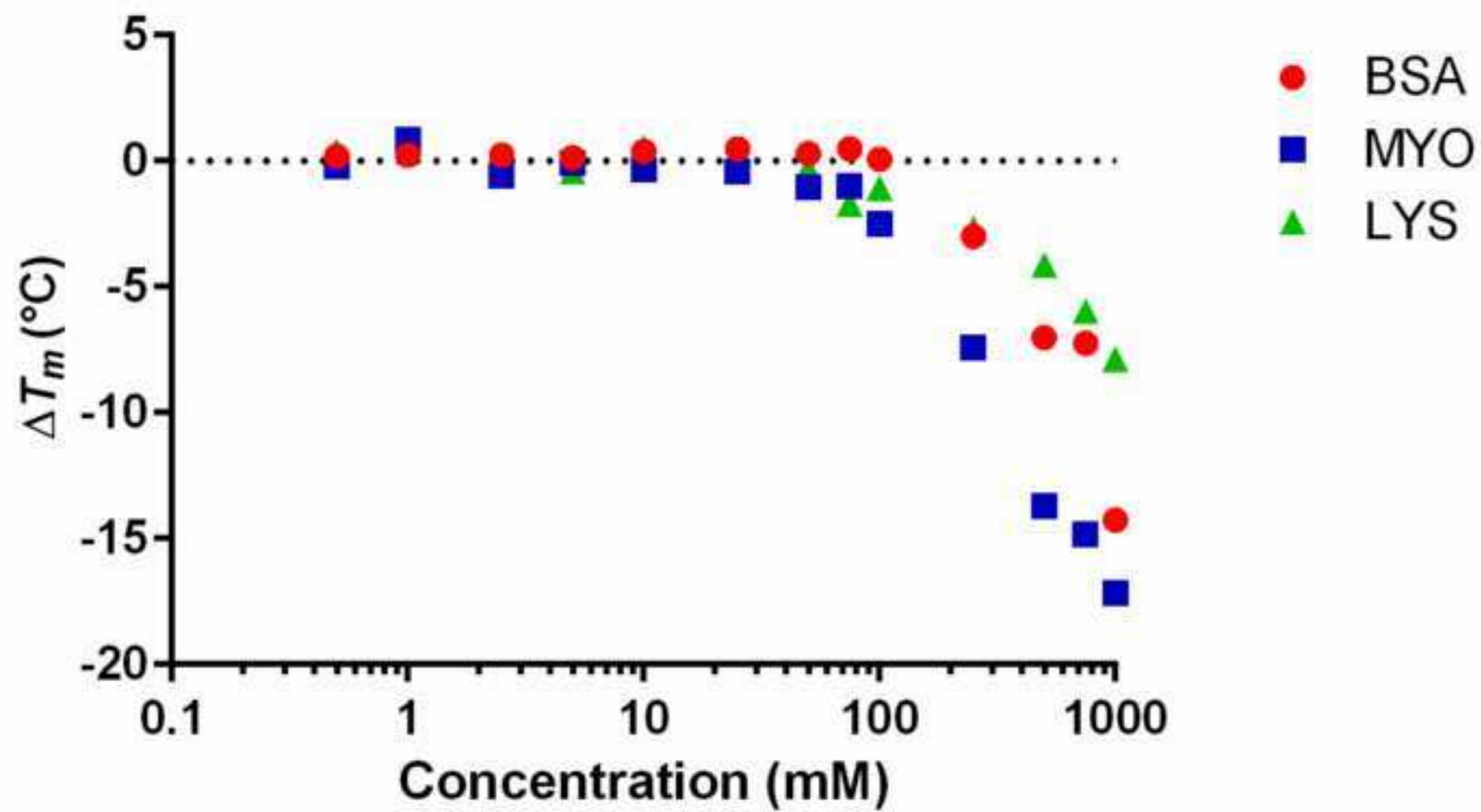
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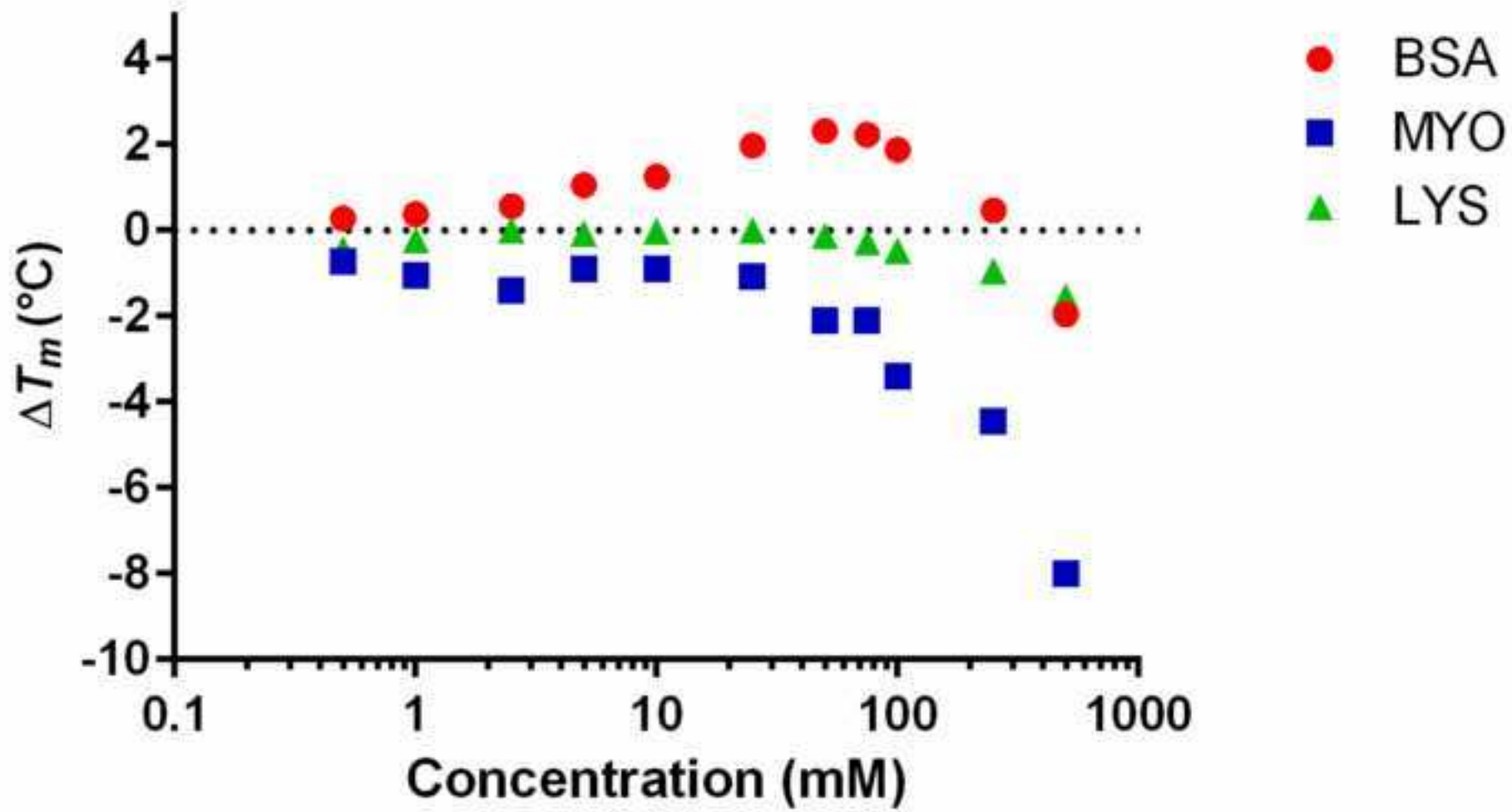
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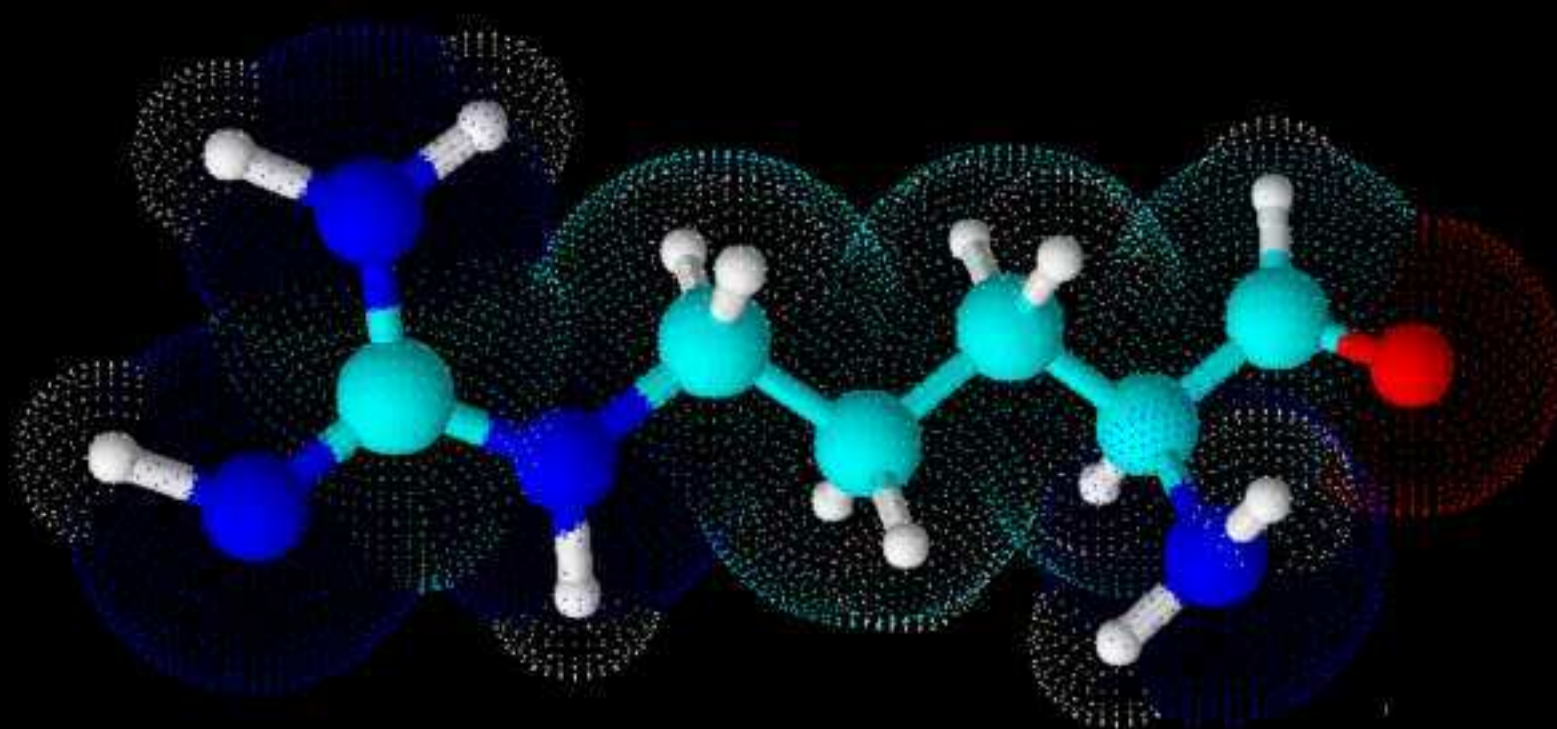


Figure(s)



Figure(s)





Guanidinium component
Mechanism 3 "Wetting agent"

Glycine component
Mechanism 1 Direct weak interaction
Mechanism 2 Competition for water

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1 **Controlling protein stability: mechanisms revealed using formulations of arginine, glycine and**
2 **guanidinium HCl with three globular proteins.**

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8 **Keywords:** calorimetry, DSC, aggregation, stabilization, formulation, osmolyte

9

10 **Abstract**

11 Three distinct interactions between the amino acid arginine and a protein explain arginine's ability
12 to modulate the thermal stability of proteins. Arginine's effect on the protein unfolding behaves like
13 the sum of its constituent parts, glycine and the guanidinium ion. The authors propose that glycine
14 can affect the thermal stability of a protein in two ways: (1) direct interaction with the charged side
15 chains and/or the peptide backbone of the protein which is observed at low concentrations and (2)
16 competition for water between the unfolding protein and the cosolute increasing the energy
17 required to hydrate the unfolding protein. The guanidinium ion acts by (3) direct interaction with
18 apolar regions exposed during unfolding reducing the energy required to hydrate the unfolding
19 protein.

20

21 **1. Introduction**

22 In recent years amino acids and other osmotically active organic molecules have found extensive
23 application as excipients in biopharmaceutical formulations (Arakawa et al., 2007; Bye et al., 2014).
24 This is due to their apparent positive effects on the solubility and stability of proteins; a consequence
25 of their ubiquitous nature in cells of almost all organisms. One such small molecule arginine is
26 commonly used as an additive in therapeutic protein manufacture and storage due to its reported
27 ability to solubilize proteins from inclusion bodies and prevent aggregation during chromatographic
28 purification steps. Its popularity as an additive stems from the fact that it appears to increase the
29 solubility of proteins without decreasing stability (Arakawa et al., 2007), unlike traditional
30 chaotropes such as urea and guanidinium which have negative effects on stability making them
31 unsuitable excipient candidates. However, the mechanism by which arginine achieves this seemingly
32 unique effect is still open to conjecture, and despite numerous attempts a consensus has not yet
33 been reached. If the mechanisms by which arginine acts can be comprehensively described and
34 understood, its effect on proteins can be predicted more accurately making its use as an excipient
35 more reliable.

36 The structural similarity of arginine's side chain to guanidinium has been linked to its ability to act
37 like a chaotropic salt in terms of solubilizing proteins and inhibiting aggregation (Xie et al., 2004).
38 However, differences in their effects on protein solubility and stability have prompted alternative
39 theories (Ishibashi et al., 2005). One point that has largely been ignored is the role other constituent
40 groups of the arginine molecule play in its action on protein stability, specifically the peptide end
41 which closely resembles a glycine molecule. Glycine is another commonly used amino acid in protein
42 formulations and a well-known stabilizer. Arginine's effect on structural stability of proteins appears
43 to vary depending on the protein studied, with no effect seen on the melting temperature of
44 lysozyme or RNase A (Arakawa et al., 2007; Reddy et al., 2005) but varying effects on that of
45 monoclonal antibodies (Thakkar et al., 2012), which has made it much harder to establish a
46 molecular mechanism for arginine's action. A lack of data reflecting the effect of lower
47 concentrations (<100 mM) of arginine on protein stability has also contributed to the absence of a

48 consensus. This is despite excipients regularly being used at these low concentrations, which are also
49 physiologically relevant. Hofmeister salts have recently been shown to affect protein stability via
50 different mechanisms at concentrations below 10 mM (Bye and Falconer 2013; Bye and Falconer
51 2014), which brings to question whether more complex small molecules follow a similar trend.
52 Furthermore, structural stability studies of proteins in the presence of additives are routinely carried
53 out in solutions with salt-containing buffers, which are also known to have an effect on protein
54 stability. These buffers could mask or enhance the effect of the additives by competitively binding to
55 charged amino acid side chains on the protein therefore blocking the binding of the arginine.

56 The aim of this study is to quantify the effects of arginine on the thermal stability of three well
57 characterized proteins, particularly at low concentrations (<100 mM) and in the absence of
58 potentially competing buffers and salts. The study will challenge the hypothesis that arginine
59 effectively acts as a guanidinium hydrochloride and a glycine linked by a three carbon chain linker. A
60 comparison of arginine effects to those of both guanidinium hydrochloride and glycine should bring
61 to light how the different structural components of the arginine molecule contribute to its actions
62 on protein stability.

63 **2. Materials and methods**

64 Bovine serum albumin (BSA), myoglobin from equine skeletal muscle & lysozyme from chicken egg
65 white, sodium octanoate and the cosolutes L-arginine, glycine and guanidinium hydrochloride
66 (GdnHCl) were all purchased from Sigma-Aldrich. The proteins were dissolved in and dialyzed against
67 HPLC grade water overnight at 4 °C using a Mini 8 kDa membrane dialysis kit (GE Healthcare, Little
68 Chalfont, UK) prior to experiments being carried out. 10 mM sodium octanoate was added to the
69 BSA solution as the protein was unstable in its absence, which should not have an effect on the
70 interaction with the cosolutes due to the specificity of its binding. Concentrations of 1 g BSA/l, 1 g
71 Lysozyme/l and 0.5 g Myoglobin/l were used at pH 7, and 8 respectively. These proteins were chosen
72 as they follow a two-state transition during unfolding, which is necessary to analyze the data and

73 they also represent a selection of differing net charges and sizes. Stocks of the cosolutes were made
74 to 2 M for glycine and guanidinium, and 1 M arginine, representing its solubility limit, and were also
75 adjusted to the appropriate pH.

76 Thermal stability of the proteins was established by Differential Scanning Calorimetry (DSC) using a
77 Nano-DSC (TA Instruments, New Castle, DE, USA) at a scan rate of 1.5 °C/min from 30 °C to 100 °C.
78 Software provided with the equipment was used to analyze the data, water-water baselines were
79 subtracted from the sample data and the temperature of maximum unfolding of the protein (T_m)
80 was calculated as the point at which the maximum relative heat capacity occurred. DSC scans of
81 each protein run repeatedly throughout the day show a precision in T_m of 0.23 °C for BSA, 0.41 °C for
82 myoglobin and 0.31 °C for lysozyme.

83 **3. Results and discussion**

84 The relative change in the T_m values of the proteins with the addition of increasing concentrations of
85 the cosolutes (arginine, glycine and guanidinium HCl) were calculated from DSC scans (Figure 1). At a
86 concentration of 1 M, glycine stabilized the proteins (Figure 2a) and guanidinium hydrochloride
87 destabilized at the same concentration (Figure 2b), as previously described (Bruździak et al., 2013;
88 Arakawa and Timasheff 1983) . At a concentration of 500 mM, arginine also appears to destabilize,
89 although not as strongly as guanidinium (Figure 2c), as previously described (Xie et al., 2004;
90 Ishibashi et al., 2005). For all three cosolutes their effects are more pronounced above 100 mM
91 (Figure 2) and these effects are similar for the three proteins, meaning the three cosolutes exert the
92 same effect regardless of protein charge and size. This is particularly apparent as lysozyme is
93 positively charged at pH 7, and both BSA and myoglobin are negatively charged at pH 7 & 8
94 respectively, yet there is no discernible difference in trend between the differently charged proteins
95 above 100 mM, and lysozyme sits between BSA and myoglobin for both arginine and glycine at
96 cosolute concentrations below 100 mM (Figure 2a&c). As a result electrostatic interactions can be
97 ruled out as a major mechanism for interaction in this case; as arginine and guanidinium

98 hydrochloride are positively charged molecules you would expect their effects to be more
99 pronounced for the oppositely charged BSA and myoglobin.

100 *3.1 Glycine*

101 Glycine stabilized all three proteins at glycine concentrations above 100 mM as previously reported
102 (Khan et al., 2013; Santoro et al., 1992). Below 100 mM more protein-specific effects are seen. At 50
103 mM glycine BSA T_m value was increased by 0.9 °C and lysozyme was relatively unaffected with a T_m
104 value just 0.4 °C above pure water. Myoglobin on the other hand was destabilized by the presence of
105 glycine with a reduction of the T_m value by 1.0 °C (Figure 2a). This has not been recorded in previous
106 publications. DSC scans of BSA in the presence of increasing concentrations of glycine demonstrate
107 that the change in heat capacity between the folded (pre-peak) and unfolded (post-peak) protein is
108 unaffected by the presence of glycine (Figure 1a). This suggests that direct interaction between the
109 glycine and the apolar interior of the protein exposed on unfolding is unlikely as you would expect to
110 see a difference in the change in heat capacity.

111 The T_m values for glycine at different concentrations suggest two phases with distinct mechanisms
112 that affect the thermal stability of proteins. A single mechanism such as preferential hydration
113 cannot explain the capability to destabilize then stabilize a protein as seen with myoglobin.

114 Kosmotropic anions have also been shown to have multiple concentration-dependent mechanisms
115 of action on thermal stability of lysozyme (Bye and Falconer 2014). These were attributed to
116 interaction with charged side chains at low concentrations, interaction with the peptide backbone at
117 intermediate concentrations and competition for water between the unfolding protein and the salt
118 at higher concentrations.

119 Glycine being a zwitterion has the ability to interact with the protein via both its negative and
120 positive charged groups, and due to its small size it is not sterically hindered from binding to multiple
121 parts of the protein. The number of potential sites for these weak interactions will vary depending

122 on the shape and charge of the protein, which results in the differences between glycine's effects on
123 the stability of the three proteins below 100 mM. In the case of glycine the destabilization observed
124 for the negatively charged myoglobin was not observed for the negatively charged BSA suggesting
125 that if there is an interaction with charged side chains it did not have a consistent effect on thermal
126 stability. The authors suggest that at concentrations below 100 mM the effect of glycine on protein
127 thermal stability could be attributed to interaction with the peptide backbone. It is well known that
128 interactions or repulsions with the peptide backbone play a major role in protein stabilization (Liu et
129 al., 1995; Street et al., 2006).

130 At concentrations above 100 mM glycine stabilizes all three proteins. This can be attributed to
131 competition for water between the unfolding protein and the cosolutes, similar to that seen for salts
132 (Bye and Falconer 2014). Both competition for water and preferential hydration (Timasheff 2002))
133 can be used to explain the increase in thermal stability in terms of an increase in energy required to
134 hydrate the protein as it unfolds. The competition for water theory suggests that the protein has to
135 compete with the cosolute for the water while preferential hydration suggests the phenomenon can
136 be expressed in terms of water molecules being preferentially bound to the protein due to the
137 presence of the cosolute in the bulk phase. The concentration-dependent stabilization seen here
138 corresponds to the competition for water theory. The authors suggest that below 100 mM glycine
139 there is still free bulk water present that can hydrate the newly exposed core of the protein upon
140 unfolding, which is why stabilization is unaffected by this mechanism at low concentrations. Once
141 the concentration of glycine in the bulk reaches 100 mM the protein has to compete for the water
142 that is 'locked up' in the hydration layer of glycine resulting in stabilization of the protein. The
143 'enhanced' structured water in the hydration shell of glycine has previously been seen using FTIR
144 and been used as an explanation for protein stabilization (Bruździak et al., 2013).

145 The protein-specific and largely unpredictable effect of glycine at concentrations below 100 mM is
146 particularly important as glycine is used in biopharmaceutical formulations and is naturally present

147 as an osmolyte in cells at these low concentrations. Destabilization by osmolytes has recently been
148 documented under a variety of conditions (Singh et al., 2011), and destabilization at low
149 concentrations followed by stabilization at higher concentrations has been seen with polyols and
150 glucose (Romero et al., 2009). The fact that this trend has not been observed before with amino
151 acids could simply be an artefact of the scarcity of data for protein stability in presence of osmolytes
152 at concentrations below 100 mM. There is also a tendency for researchers to use salt-containing
153 buffers in protein stability experiments, which could be screening these effects at lower
154 concentrations. Therefore this phenomenon may actually be true for many if not all osmolytes.

155 *3.2 Guanidinium Hydrochloride*

156 Guanidinium hydrochloride destabilized all three proteins by up to 17 °C between 100 mM and 1 M
157 as expected due to its well-known chaotropic action on proteins (Figure 2b). Below 100 mM
158 guanidinium hydrochloride has no notable effect on the melting temperature of any of the three
159 proteins meaning its denaturing effect only occurs at higher concentrations, which makes its
160 mechanistic action harder to explain. It is generally agreed upon that guanidine binds to the protein
161 (Courtenay et al., 2001; Lee et al., 1974; Möglich et al., 2005). Most studies suggest that this is via
162 hydrogen bonding to the peptide backbone (Street et al., 2006; Robinson et al., 1965). However
163 recent work by Lim et al. (2009) using measurement of acid and base catalyzed hydrogen exchange
164 by NMR has suggested that, although most denaturants act via this mechanism, guanidinium
165 hydrochloride does not. DSC scans of the unfolding of BSA in the presence of guanidinium
166 hydrochloride show different patterns of unfolding and an increase in the heat capacity of the
167 unfolded state compared to the folded (Figure 1b), which suggests that the guanidinium cation is
168 binding to the protein. Here we suggest that guanidinium acts like a detergent; the weakly hydrated
169 cation is pushed onto apolar parts of the protein due to strong water-water interactions, as
170 originally proposed by Collins (1995). This reduces the energy required to hydrate the apolar core of
171 the protein exposed during unfolding, resulting in a destabilization of the protein. As this is a weak

172 interaction governed mainly by water-water interactions, the destabilizing effect on the protein is
173 not seen until guanidinium hydrochloride is present at concentrations above 100 mM.

174 *3.3 Arginine*

175 Below 100 mM arginine acts very similarly to glycine in that protein-specific stabilization of BSA,
176 destabilization of myoglobin and neutral effects on lysozyme are seen. Above 100mM it begins to
177 destabilize all three proteins in a similar manner to the guanidinium, although not as strongly (Figure
178 2c). Arginine's mechanism of action is thought to be complex, as demonstrated by varying effects on
179 protein stability and concentration-dependent actions (Thakkar et al., 2012; Falconer et al., 2011).
180 However, current suggestions of weak transient interactions at low concentrations (Lim et al., 2009)
181 and preferential exclusion due to increase in surface tension and self-association or 'stacking' of
182 arginine molecules at higher concentrations (Shukla and Trout 2011; Das et al., 2007; Kita et al.,
183 1994; Vondrášek et al., 2009) cannot be used to explain the trends presented in this paper as this
184 would result in a stronger stabilization at higher concentrations. Instead we propose that arginine is
185 indeed acting as a mixture of its structural components and that the mechanisms for glycine and
186 guanidinium hydrochloride also explain arginine's functionality. The authors propose that the
187 confusion about arginine is due to the fact that it can affect the thermal stability of a protein in
188 multiple ways including the interaction with charged side chains and/or the peptide backbone,
189 competition for water (between the unfolding protein and the cosolute) and the interaction with
190 apolar regions exposed during unfolding (here referred to as a "wetting agent" effect). At
191 concentrations below 100 mM the overriding effect of arginine on protein stability is governed by its
192 glycine group, which is interacting weakly with the peptide backbone and exposed polar groups as
193 explained in section 3.1 causing a protein-specific effect. Above 100 mM the denaturing action of the
194 guanidinium group comes into effect as described in section 3.3, which involves the arginine
195 molecules being forced onto apolar regions of the protein, causing unfolding to be more favorable
196 due to it being energetically easier to hydrate the hydrophobic core of the protein. The strength of

197 the destabilization by arginine above 100 mM is weaker than that of the guanidinium alone, which
198 can be explained by the competing effects of the guanidinium and glycine portions of the arginine
199 molecule simultaneously competing for water, which increases the free energy of unfolding, and
200 having a wetting agent effect, which reduces the free energy of unfolding.

201 *3.4 Implications for formulation design*

202 The importance of using reliable excipients in biopharmaceutical formulations to stabilize
203 therapeutic proteins that would otherwise be unstable must not be underestimated. Both arginine
204 and glycine are regularly used as stabilizing excipients in biopharmaceutical formulations at
205 concentrations below 100 mM (Parkins et al., 2000). The data shown here demonstrates that both
206 small molecules can act in a more complex manner than first thought, with trends that exhibit
207 concentration-dependence and protein-specificity. Both of these characteristics could potentially
208 prevent arginine and glycine from being ideal excipient candidates as it is difficult to predict under
209 which specific conditions they exert the desired stabilizing effect. Based on the finding that arginine
210 acts like a combination of its constituent groups, the authors suggest using a mixture of glycine and
211 guanidinium hydrochloride in the place of arginine in formulations. This presents the advantage of
212 being able to specifically alter the ratios of the stabilizing glycine molecule and the solubilizing and
213 aggregation-preventing guanidinium molecule, in order to more accurately create the optimum
214 conditions for long-term storage of therapeutic proteins in aqueous solutions.

215 **4. Conclusions**

216 We have shown that arginine acts on protein stability like a combination of its constituent groups.
217 Below 100 mM arginine acts like glycine, above 100 mM it shows destabilizing effects similar to
218 guanidinium hydrochloride. The glycine alone demonstrates two stages of stabilization. The first
219 effect (at concentrations below 100 mM) is protein specific and is probably due to multiple direct
220 interactions with the polar or charged side chains and the partial charges on the peptide backbone
221 of the protein. The second stage (at concentrations above 100 mM) is similar to high charge density

222 anions where it was ascribed to competition for water between the unfolding (Bye and Falconer
223 2014). Guanidinium hydrochloride acts by direct interaction with apolar regions exposed as the
224 protein unfolds, which we call a “wetting agent” effect reducing the free energy required to hydrate
225 the protein’s interior as it unfolds.

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300

301

302 **Figure Titles**

303

304 Figure 1. Representative DSC Scans of 1g/l BSA with 10mM sodium octanoate at pH 7 with different
305 concentrations of (a) glycine, (b) guanidinium hydrochloride and (c) arginine to show changes in T_m
306 represented by highest point of relative heat capacity and changes to unfolding pattern. BSA without
307 additives is plotted in each panel for comparison. A water baseline has been subtracted and initial
308 heat capacities have been normalized.

309 Figure 2. Relative change in the T_m of 1 g/L BSA at pH 7 (circles), 1 g/L lysozyme at pH 7 (triangles)
310 and 0.5 g/L myoglobin at pH 8 (squares) between the protein solution with no additives present and
311 in the presence of increasing concentrations of (a) glycine, (b) guanidinium hydrochloride and (c)
312 arginine plotted on a logarithmic scale. Dotted line shows no relative change in the T_m of the protein
313 to guide the eye.