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

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Abstract

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

1. Introduction

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

The structural similarity of arginine's side chain to guanidinium has been linked to its ability to act like a chaotropic salt in terms of solubilizing proteins and inhibiting aggregation (Xie et al., 2004). However, differences in their effects on protein solubility and stability have prompted alternative theories (Ishibashi et al., 2005). One point that has largely been ignored is the role other constituent groups of the arginine molecule play in its action on protein stability, specifically the peptide end which closely resembles a glycine molecule. Glycine is another commonly used amino acid in protein formulations and a well-known stabilizer. Arginine's effect on structural stability of proteins appears to vary depending on the protein studied, with no effect seen on the melting temperature of lysozyme or RNase A (Arakawa et al., 2007; Reddy et al., 2005) but varying effects on that of monoclonal antibodies (Thakkar et al., 2012), which has made it much harder to establish a molecular mechanism for arginine's action. A lack of data reflecting the effect of lower concentrations (<100 mM) of arginine on protein stability has also contributed to the absence of a consensus. This is despite excipients regularly being used at these low concentrations, which are also physiologically relevant. Hofmeister salts have recently been shown to affect protein stability via different mechanisms at concentrations below 10 mM (Bye and Falconer 2013; Bye and Falconer 2014), which brings to question whether more complex small molecules follow a similar trend. Furthermore, structural stability studies of proteins in the presence of additives are routinely carried out in solutions with salt-containing buffers, which are also known to have an effect on protein stability. These buffers could mask or enhance the effect of the additives by competitively binding to charged amino acid side chains on the protein therefore blocking the binding of the arginine.

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

2. Materials and methods

Bovine serum albumin (BSA), myoglobin from equine skeletal muscle & lysozyme from chicken egg white, sodium octanoate and the cosolutes L-arginine, glycine and guanidinium hydrochloride (GdnHCl) were all purchased from Sigma-Aldrich. The proteins were dissolved in and dialyzed against HPLC grade water overnight at 4 °C using a Mini 8 kDa membrane dialysis kit (GE Healthcare, Little Chalfont, UK) prior to experiments being carried out. 10 mM sodium octanoate was added to the BSA solution as the protein was unstable in its absence, which should not have an effect on the interaction with the cosolutes due to the specificity of its binding. Concentrations of 1 g BSA/l, 1 g Lysozyme/l and 0.5 g Myoglobin/l were used at pH 7, and 8 respectively. These proteins were chosen as they follow a two-state transition during unfolding, which is necessary to analyze the data and they also represent a selection of differing net charges and sizes. Stocks of the cosolutes were made to 2 M for glycine and guanidinium, and 1 M arginine, representing its solubility limit, and were also adjusted to the appropriate pH.

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

3. Results and discussion

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

3.1 Glycine

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

The T_m values for glycine at different concentrations suggest two phases with distinct mechanisms that affect the thermal stability of proteins. A single mechanism such as preferential hydration cannot explain the capability to destabilize then stabilize a protein as seen with myoglobin. Kosmotropic anions have also been shown to have multiple concentration-dependent mechanisms of action on thermal stability of lysozyme (Bye and Falconer 2014). These were attributed to interaction with charged side chains at low concentrations, interaction with the peptide backbone at intermediate concentrations and competition for water between the unfolding protein and the salt at higher concentrations.

Glycine being a zwitterion has the ability to interact with the protein via both its negative and positive charged groups, and due to its small size it is not sterically hindered from binding to multiple parts of the protein. The number of potential sites for these weak interactions will vary depending on the shape and charge of the protein, which results in the differences between glycine's effects on the stability of the three proteins below 100 mM. In the case of glycine the destabilization observed for the negatively charged myoglobin was not observed for the negatively charged BSA suggesting that if there is an interaction with charged side chains it did not have a consistent effect on thermal stability. The authors suggest that at concentrations below 100 mM the effect of glycine on protein thermal stability could be attributed to interaction with the peptide backbone. It is well known that interactions or repulsions with the peptide backbone play a major role in protein stabilization (Liu et al., 1995; Street et al., 2006).

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

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

3.2 Guanidinium Hydrochloride

Guanidinium hydrochloride destabilized all three proteins by up to 17 °C between 100 mM and 1 M as expected due to its well-known chaotropic action on proteins (Figure 2b). The change in T_m value was not proportional to the denaturant concentration as predicted from previous work on the apparent free energy of unfolding in the presence of guanidinium hydrochloride (Greene and Pace 1974). Deviation from a linear relationship was particularly pronounced for BSA and myoglobin. Below 100 mM guanidinium hydrochloride has no notable effect on the melting temperature of any of the three proteins meaning its denaturing effect only occurs at higher concentrations, which makes its mechanistic action harder to explain. It is generally agreed upon that guanidinium binds to the protein (Courtenay et al., 2001; Lee et al., 1974; Möglich et al., 2005). Most studies suggest that this is via hydrogen bonding to the peptide backbone (Street et al., 2006; Robinson et al., 1965). However, recent work by Lim et al. (2009) using measurement of acid and base catalyzed hydrogen exchange by NMR has suggested that, although most denaturants act via this mechanism, guanidinium hydrochloride does not. DSC scans of the unfolding of BSA in the presence of guanidinium hydrochloride show different patterns of unfolding and an increase in the heat capacity of the unfolded state compared to the folded (Figure 1b), which suggests that the guanidinium cation is binding to the protein. Here we suggest that guanidinium acts like a detergent; the weakly hydrated cation is pushed onto apolar parts of the protein due to strong water-water interactions, as originally proposed by Collins (1995). This reduces the energy required to hydrate the apolar core of the protein exposed during unfolding, resulting in a destabilization of the protein. As this is a weak interaction governed mainly by water-water interactions, the destabilizing effect on the protein is not seen until guanidinium hydrochloride is present at concentrations above 100 mM.

3.3 Arginine

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

3.4 Implications for formulation design

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

4. Conclusions

We have shown that arginine acts on protein stability like a combination of its constituent groups. Below 100 mM arginine acts like glycine, above 100 mM it shows destabilizing effects similar to guanidinium hydrochloride. The glycine alone demonstrates two stages of stabilization. The first effect (at concentrations below 100 mM) is protein specific and is probably due to multiple direct interactions with the polar or charged side chains and the partial charges on the peptide backbone of the protein. The second stage (at concentrations above 100 mM) is similar to high charge density anions where it was ascribed to competition for water between the unfolding (Bye and Falconer 2014). Guanidinium hydrochloride acts by direct interaction with apolar regions exposed as the protein unfolds, which we call a “wetting agent” effect reducing the free energy required to hydrate the protein’s interior as it unfolds.

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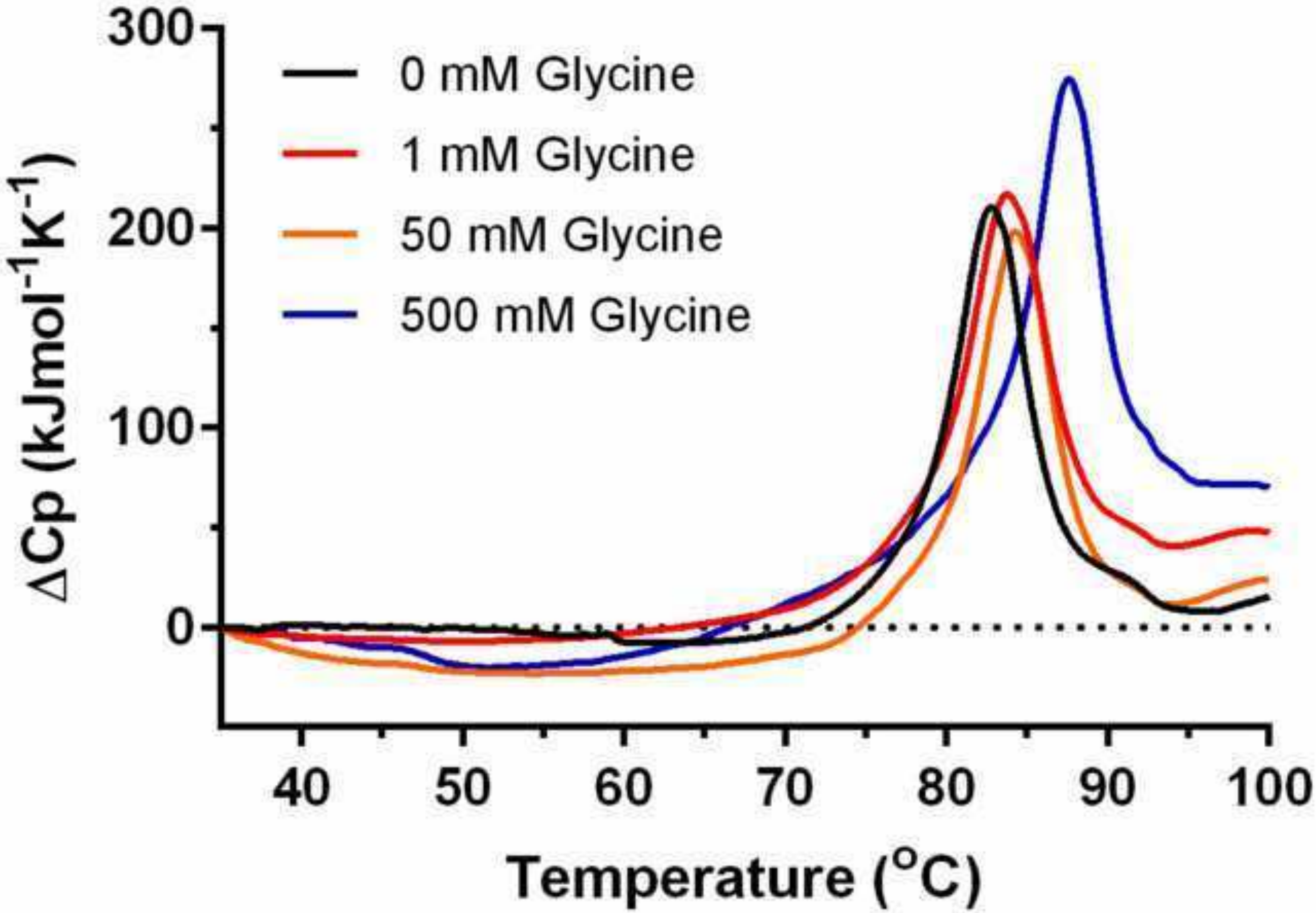
Figure Titles

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

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) and 0.5 g/L myoglobin at pH 8 (squares) between the protein solution with no additives present and in the presence of increasing concentrations of (a) glycine, (b) guanidinium hydrochloride and (c)

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

Figure(s)



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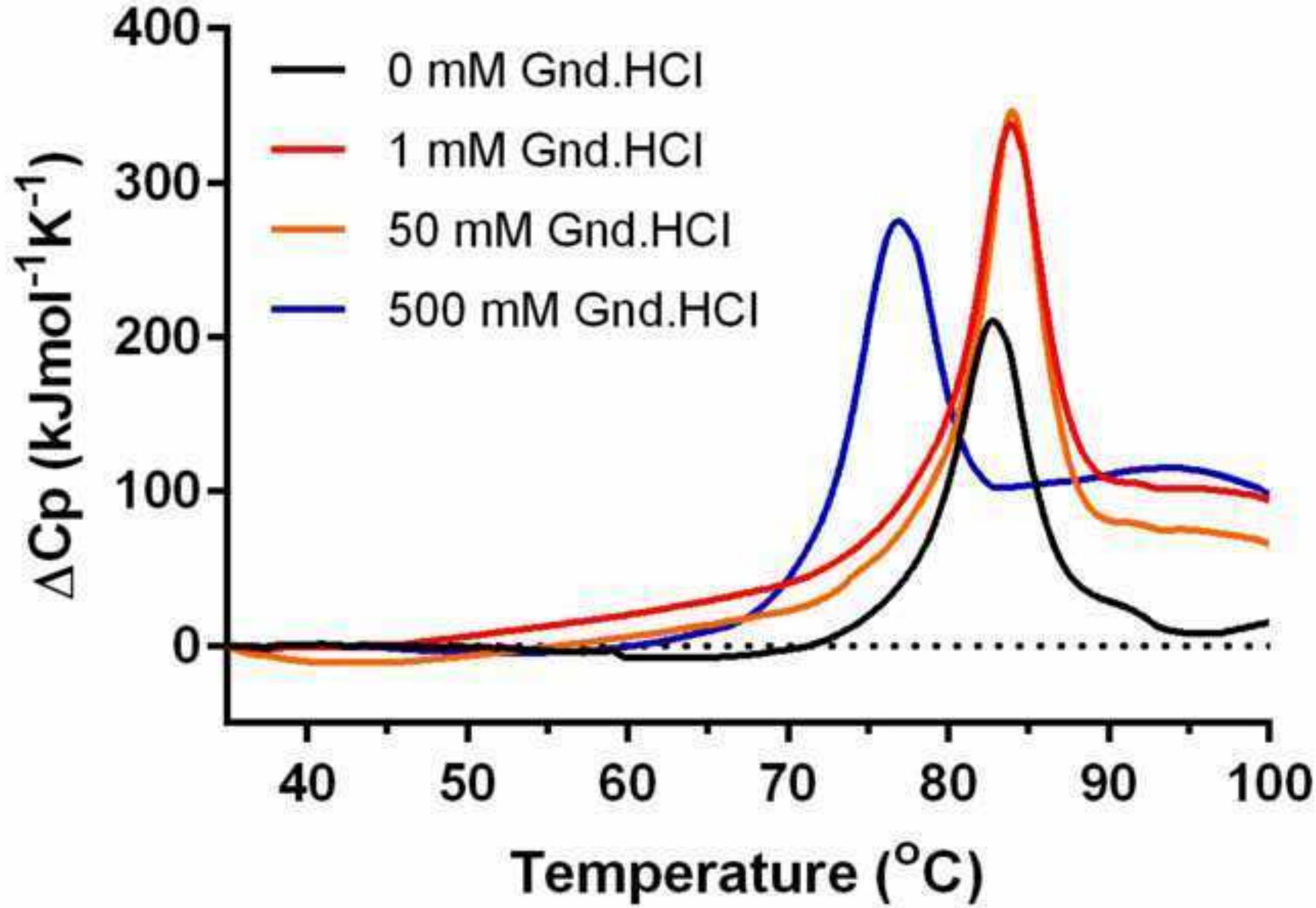
Temperature (°C)

Figure(s)



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ΔCp (kJmol-1K-1)
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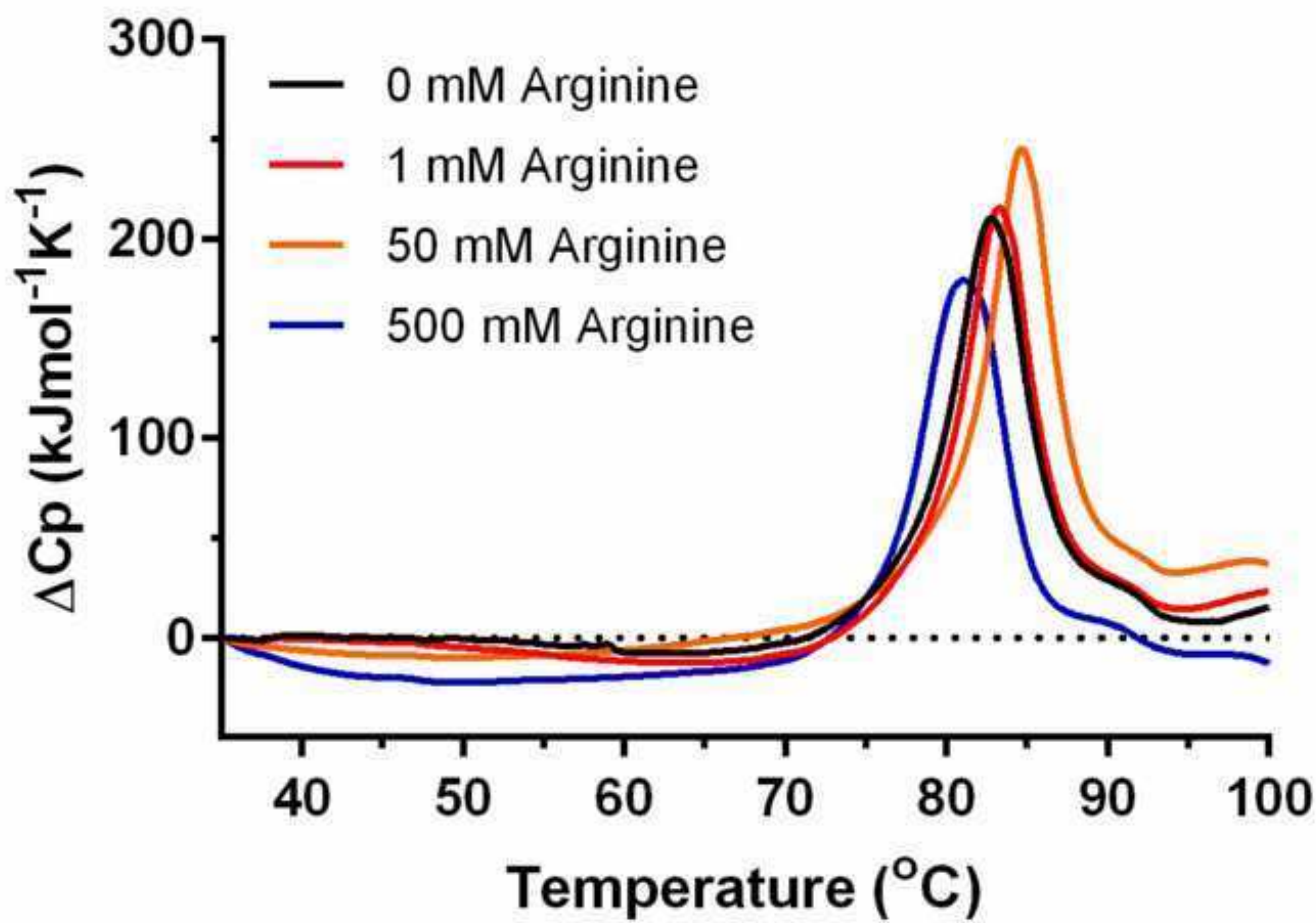
Temperature (°C)

Figure(s)



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ΔCp (kJmol-1K-1)
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Temperature (°C)
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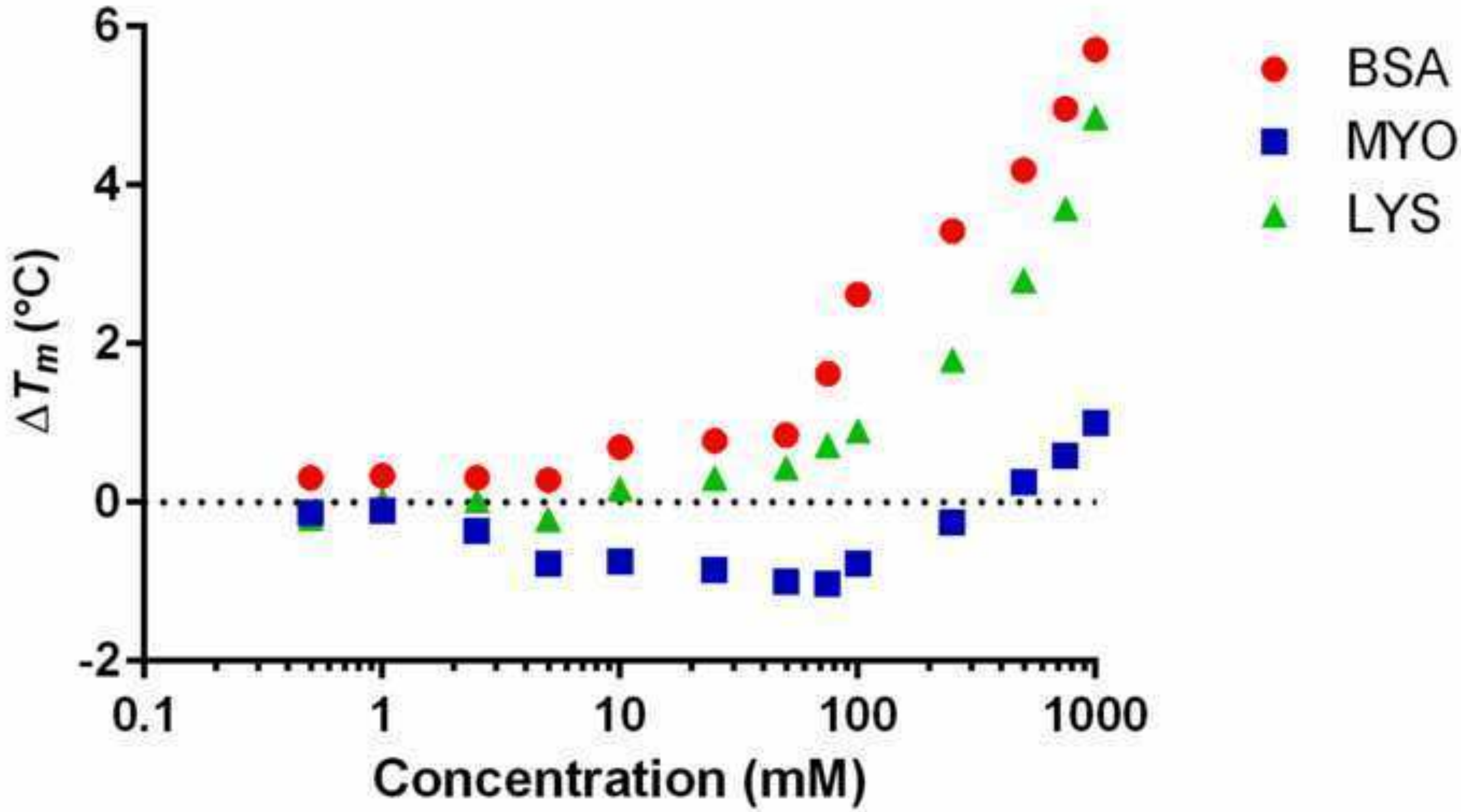
500 mM Arginine

Figure(s)



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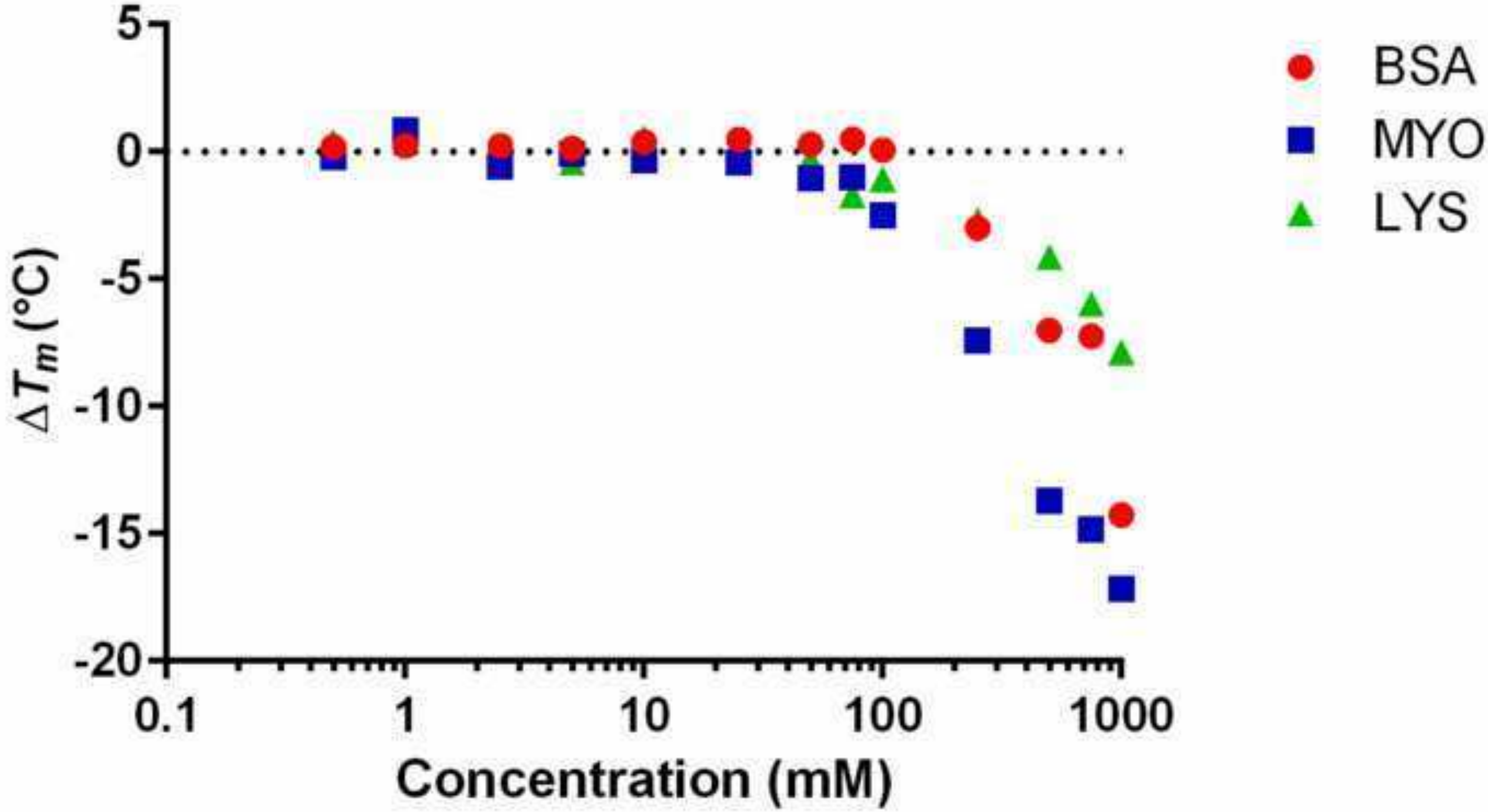

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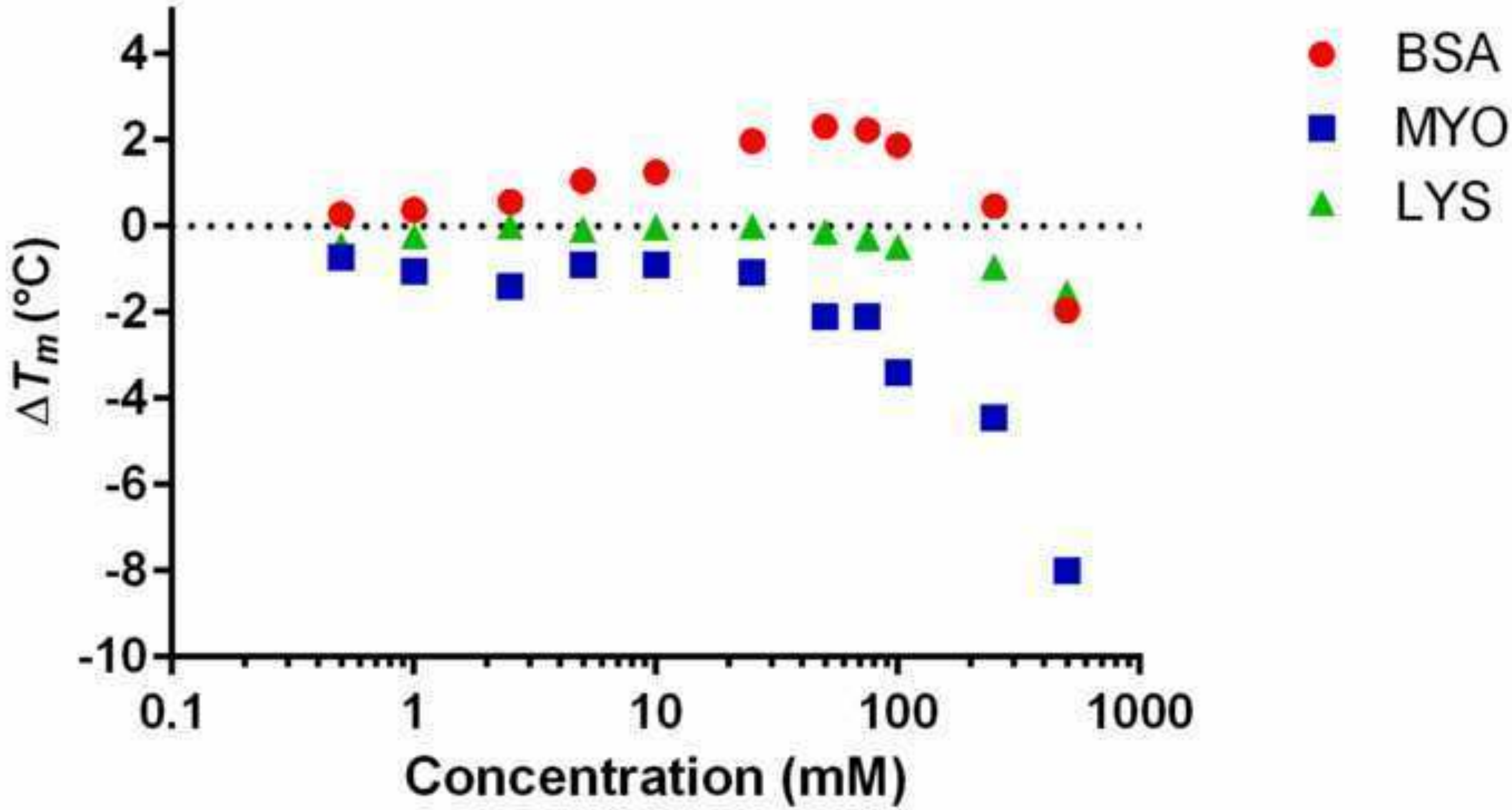
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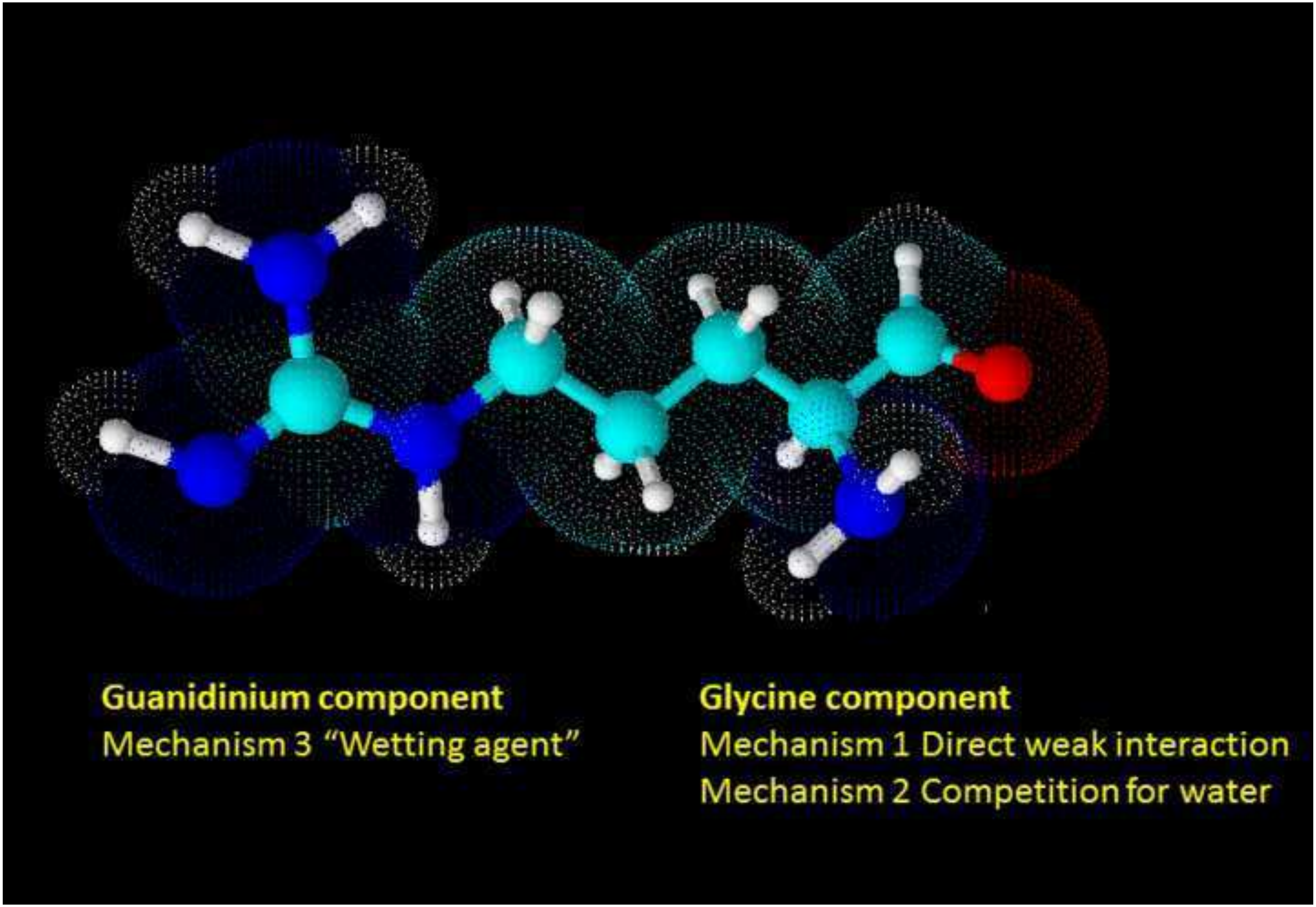

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Controlling protein stability: mechanisms revealed using formulations of arginine, glycine and guanidinium HCl with three globular proteins.

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

Abstract

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

1. Introduction

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

The structural similarity of arginine’s side chain to guanidinium has been linked to its ability to act like a chaotropic salt in terms of solubilizing proteins and inhibiting aggregation (Xie et al., 2004). However, differences in their effects on protein solubility and stability have prompted alternative theories (Ishibashi et al., 2005). One point that has largely been ignored is the role other constituent groups of the arginine molecule play in its action on protein stability, specifically the peptide end which closely resembles a glycine molecule. Glycine is another commonly used amino acid in protein formulations and a well-known stabilizer. Arginine’s effect on structural stability of proteins appears to vary depending on the protein studied, with no effect seen on the melting temperature of lysozyme or RNase A (Arakawa et al., 2007; Reddy et al., 2005) but varying effects on that of monoclonal antibodies (Thakkar et al., 2012), which has made it much harder to establish a molecular mechanism for arginine’s action. A lack of data reflecting the effect of lower concentrations (<100 mM) of arginine on protein stability has also contributed to the absence of a consensus. This is despite excipients regularly being used at these low concentrations, which are also physiologically relevant. Hofmeister salts have recently been shown to affect protein stability via different mechanisms at concentrations below 10 mM (Bye and Falconer 2013; Bye and Falconer 2014), which brings to question whether more complex small molecules follow a similar trend. Furthermore, structural stability studies of proteins in the presence of additives are routinely carried out in solutions with salt-containing buffers, which are also known to have an effect on protein stability. These buffers could mask or enhance the effect of the additives by competitively binding to charged amino acid side chains on the protein therefore blocking the binding of the arginine.

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

2. Materials and methods

Bovine serum albumin (BSA), myoglobin from equine skeletal muscle & lysozyme from chicken egg white, sodium octanoate and the cosolutes L-arginine, glycine and guanidinium hydrochloride (GdnHCl) were all purchased from Sigma-Aldrich. The proteins were dissolved in and dialyzed against HPLC grade water overnight at 4 °C using a Mini 8 kDa membrane dialysis kit (GE Healthcare, Little Chalfont, UK) prior to experiments being carried out. 10 mM sodium octanoate was added to the BSA solution as the protein was unstable in its absence, which should not have an effect on the interaction with the cosolutes due to the specificity of its binding. Concentrations of 1 g BSA/l, 1 g Lysozyme/l and 0.5 g Myoglobin/l were used at pH 7, and 8 respectively. These proteins were chosen as they follow a two-state transition during unfolding, which is necessary to analyze the data and they also represent a selection of differing net charges and sizes. Stocks of the cosolutes were made to 2 M for glycine and guanidinium, and 1 M arginine, representing its solubility limit, and were also adjusted to the appropriate pH.

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

3. Results and discussion

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

3.1 Glycine

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

The T_m values for glycine at different concentrations suggest two phases with distinct mechanisms that affect the thermal stability of proteins. A single mechanism such as preferential hydration cannot explain the capability to destabilize then stabilize a protein as seen with myoglobin. Kosmotropic anions have also been shown to have multiple concentration-dependent mechanisms of action on thermal stability of lysozyme (Bye and Falconer 2014). These were attributed to interaction with charged side chains at low concentrations, interaction with the peptide backbone at intermediate concentrations and competition for water between the unfolding protein and the salt at higher concentrations.

Glycine being a zwitterion has the ability to interact with the protein via both its negative and positive charged groups, and due to its small size it is not sterically hindered from binding to multiple parts of the protein. The number of potential sites for these weak interactions will vary depending on the shape and charge of the protein, which results in the differences between glycine's effects on the stability of the three proteins below 100 mM. In the case of glycine the destabilization observed for the negatively charged myoglobin was not observed for the negatively charged BSA suggesting that if there is an interaction with charged side chains it did not have a consistent effect on thermal stability. The authors suggest that at concentrations below 100 mM the effect of glycine on protein thermal stability could be attributed to interaction with the peptide backbone. It is well known that interactions or repulsions with the peptide backbone play a major role in protein stabilization (Liu et al., 1995; Street et al., 2006).

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

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

3.2 Guanidinium Hydrochloride

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

3.3 Arginine

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

3.4 Implications for formulation design

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

4. Conclusions

We have shown that arginine acts on protein stability like a combination of its constituent groups. Below 100 mM arginine acts like glycine, above 100 mM it shows destabilizing effects similar to guanidinium hydrochloride. The glycine alone demonstrates two stages of stabilization. The first effect (at concentrations below 100 mM) is protein specific and is probably due to multiple direct interactions with the polar or charged side chains and the partial charges on the peptide backbone of the protein. The second stage (at concentrations above 100 mM) is similar to high charge density anions where it was ascribed to competition for water between the unfolding (Bye and Falconer 2014). Guanidinium hydrochloride acts by direct interaction with apolar regions exposed as the protein unfolds, which we call a “wetting agent” effect reducing the free energy required to hydrate the protein’s interior as it unfolds.

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Figure Titles

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

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) and 0.5 g/L myoglobin at pH 8 (squares) between the protein solution with no additives present and in the presence of increasing concentrations of (a) glycine, (b) guanidinium hydrochloride and (c) arginine plotted on a logarithmic scale. Dotted line shows no relative change in the T_m of the protein to guide the eye.