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Control of globular protein thermal stability in aqueous formulations by the positively charged amino acid excipients

Lauren Platts, Samuel J Darby, Robert J Falconer*

Department of Chemical & Biological Engineering, ChELSI Institute, University of Sheffield, Sheffield, S1 3JD, England

* To whom the correspondence should be addressed. Telephone +44 114 2228253, Fax +44 114 2227501 Email r.j.falconer@sheffield.ac.uk

The positively charged amino acids are commonly used excipients in biopharmaceutical formulations for stabilisation of therapeutic proteins yet the mechanisms for their modulation of protein stability are poorly understood. In this study both lysine and histidine are shown to affect the thermal stability of myoglobin, bovine serum albumin and lysozyme through a combination of mechanisms governed by their respective functional side chains and glycine, similar to arginine. This study provides evidence that at low concentrations lysine and histidine interact with proteins via (1) direct electrostatic interactions with negatively charged side chains, (2) binding to high affinity hydrophobic binding sites and (3) glycine-mediated weak interactions with peptide backbone and polar side chains. At high concentrations lysine and histidine act via (4) glycine-mediated competition for water between the unfolding protein and the excipient and (5) sidechain-mediated interaction with apolar regions exposed during unfolding (histidine) or interaction with polar regions of the protein (lysine). Lysine and histidine may prove useful for biopharmaceutical formulation scientists as they were less destabilising of the proteins tested than arginine at concentrations above 100 mM.

Keywords

Calorimetry (DSC); Protein formulation; Protein folding/refolding; Stabilization; Excipients

INTRODUCTION

Designing effective liquid formulations for biopharmaceutical products currently presents a major challenge to the pharmaceutical industry. A large part of this problem is that choosing suitable excipients to maintain stability of protein therapeutics is usually based on what has been used previously and which excipients are currently approved for use. The range of molecules that meet these criteria is limited at present.[1] Amino acids are amongst some of the most commonly used stabilising excipients as they are already naturally present in cells and so are considered to be extremely safe to use.[2]

Interestingly, usually featured in lists of commonly used excipients are the three positively charged amino acids: arginine, lysine and histidine.[1,3] Many studies have shown lysine to stabilise proteins in solution [4–7] despite the fact that it has been demonstrated to perturb enzyme function. Quite often lysine is shown to stabilise proteins to a much lesser extent than other amino acids and small molecules. This is usually attributed to a combination of direct binding to the protein and an increase in surface tension of the solution,[8] resulting in weak preferential exclusion from the protein surface.[4]

The data for the effects of histidine on protein stability are much more scarce and much less uniform, with certain studies showing a destabilising effect on proteins[5] and others showing histidine as a protein stabiliser.[7,9] No clear mechanism has yet been suggested for the effect of histidine on protein stability. In addition to being a protein stabilising excipient histidine's usefulness in protein formulations generally stems from its buffering capacity as it has a pKa of around 6.0.

The effects of arginine on protein stability on the other hand have been thoroughly studied and many different mechanisms suggested.[10–13] One such mechanism that has been proposed by multiple authors is principally based on the influence of its positively charged side chain, which is similar in structure to that of the well documented chaotrope, or protein destabiliser, guanidinium.[14,15] Similar mechanisms for both lysine and histidine do not appear to have been investigated in the literature despite the fact that molecules with structural similarity to their positively charged side chains, methylamine hydrochloride and imidazole respectively, have important pharmaceutical uses themselves. Imidazole is generally used in the purification of His-tagged proteins amongst other uses.[16] Methylamine derivatives such as betaine and sarcosine are well known to counteract the protein destabilising effects of urea.[17]

The authors of this paper previously described how the effects of arginine on protein stability mimic a combination of both its side chain, guanidinium, and the glycine moiety.[18] Through removal of salt-containing buffers, which have the ability to mask or enhance protein stability effects of other small molecules, and a detailed investigation into the effects of the lower concentrations (less than 100 mM) of the excipients in question the authors were able to demonstrate that arginine's effects on protein stability are governed by a combination of the molecular mechanisms by which both glycine and guanidinium hydrochloride act on protein stability.

The aim of this study is to quantify the effects of both lysine and histidine on the thermal stability of proteins and to challenge the hypothesis that the mechanisms by which they act are similar to those of arginine, meaning that all three positively charged amino acids exert their effects on protein stability in a similar way. In this way insights gained from the extensively studied positively charged amino acid, arginine, can be used to gain understanding from the lesser studied lysine and histidine. More specifically by comparing the effects of lysine and histidine on the thermal stability of three model proteins to the effects of their side chains, methylamine hydrochloride and imidazole respectively, and the data previously obtained for glycine,[18] the molecular mechanisms by which they act on protein stability should become clear.

EXPERIMENTAL METHODS

All proteins: bovine serum albumin (BSA), myoglobin from equine skeletal muscle & lysozyme from chicken egg white were purchased from Sigma-Aldrich. Sodium octanoate and the cosolutes glycine, lysine hydrate, methylamine hydrochloride, histidine and imidazole were also purchased from Sigma-Aldrich.

Methods follow those described in the authors' previous paper .[18] Proteins were dialysed with HPLC grade water overnight at 4 °C using a Mini 8kDa membrane dialysis kit (GE Healthcare, Little Chalfont, UK). 10 mM sodium octanoate was used instead of HPLC grade water for experiments using BSA, as the protein was unstable in its absence. Due to the specificity of its binding it should not interfere with the interactions between cosolute and protein. Concentration and pH conditions for the three proteins were as follows: 1 g/L BSA at pH 7, 1 g/L lysozyme at pH 7, 0.5 g/L myoglobin at pH 8. These model proteins were chosen based on their differing characteristics of net charge and

size as well as their two-state transition of unfolding at the conditions chosen. Stocks of 2 M glycine, 2 M methylamine hydrochloride, 2 M imidazole, 1 M lysine and 400 mM histidine were made up and put to the appropriate pH. Although data for glycine had already been collected in the previous paper, data sets were repeated here as the protein stocks were new. pH was monitored over the concentration range and was found to remain at the appropriate pH.

Thermal stability data was collected through Differential Scanning Calorimetry scans conducted under the various cosolute conditions on a Nano-DSC (TA Instruments, New Castle, DE USA) with the following settings: 1.5 °C/min scan rate from 30 °C to 100 °C. Software provided with the instrument was used to subtract a water-water baseline and calculate the midpoint of thermal unfolding (T_m). Precision data for the T_m of each protein was calculated by repeated scans throughout the day and are as follows: 0.23 °C for BSA, 0.41 °C for myoglobin, 0.31 °C for lysozyme.

RESULTS AND DISCUSSION

DSC scans were conducted for formulations of three proteins in ultrapure water plus the excipient of choice. There was no additional buffering and the pH was controlled by the protein's own buffering capacity. Examples of the scans are shown in supplementary figures 1 and 2. The relative changes in midpoint of thermal unfolding (ΔT_m) were calculated for each of the three proteins by subtracting the zero cosolute concentration midpoint of thermal unfolding from the midpoint of thermal unfolding for that particular concentration. The observed trends for lysine (figure 1) and histidine (figure 2) were then compared to chemicals similar in structure to their respective side chains and the previously published data for glycine (figure 3).[18] This was done to challenge the hypothesis formed as a result of trends seen for arginine that an amino acids functionality was the sum of its constituent part (glycine and the side chain).[18] Methylamine HCl is structurally very similar to the end of the lysine side chain and imidazole is very similar to the end of the histidine side chain.

Lysine and Methylamine Hydrochloride

For both myoglobin and BSA 0.5 mM and 1 mM lysine and methylamine HCl destabilised these proteins by approximately 2 °C (figure 1). Such a low concentration of the excipients causing a drop in T_m suggests the interaction between the excipient and the protein governing this effect has a low stoichiometry. Both myoglobin and BSA are negatively charged meaning that electrostatic interactions between the positively charged excipients and negatively charged side chains are possible. There are approximately 18 molecules of lysine/methylamine HCl for every myoglobin molecule in the 0.5 mM solution and approximately 12 exposed negatively charged amino acids, meaning that the majority of the excipient molecules will be bound to the protein. In the case of BSA there are many more exposed negatively charged amino acids so again all of the excipients are likely to be bound.

Between 0.5 and 50 mM methylamine HCl there was no effect on the thermal stability of myoglobin or lysozyme but BSA was stabilised by approximately 2 °C. BSA has evolved to have many hydrophobic binding sites capable of receiving multiple excipients .[19] The presence of high affinity binding sites on the native form of BSA will result in stabilisation of the protein, as this will not favour the denatured state until such sites are saturated. Lysine shows a similar trend but also mimics the protein-specific trends of glycine seen at these concentrations (figure 3).

Over 50 mM lysine and methylamine HCl both destabilised myoglobin and BSA (by approximately 10 °C and 3 °C respectively), but lysozyme was barely affected by the presence of both lysine and methylamine HCl. This protein-specific effect at high concentrations of excipient is not seen for other

amino acids and therefore is unique to lysine. DSC scans of BSA and myoglobin in the presence of high concentrations of lysine and methylamine HCl show an increase in heat capacity of the unfolded state (supplementary figure 1), which is known to correspond to extensive binding of the excipient to polar regions.[20,21] Hydrophobic interaction chromatography has shown that, of the three model proteins, myoglobin has the most polar regions and lysozyme has the least, which mimics the stability trends.[22] Preferential interaction data has also shown lysine to preferentially accumulate more at the surface of BSA than of lysozyme,[4] which also supports this hypothesis.

Histidine and Imidazole

The solubility of histidine limited the experimentation with this excipient to 200 mM. Like lysine and methylamine HCl, histidine and imidazole altered the thermal stability of lysozyme, myoglobin and BSA at 0.5 mM (figure 2). Again, substantial effect on protein stability at such low excipient concentrations is consistent with a strong electrostatic interaction with oppositely charged side chains though this is unlikely between the positively charged lysozyme and the positively charged excipients where the stabilisation was by less than a 1 °C.

Between 0.5 and 25 mM histidine and imidazole had little measurable effect on the thermal stability of lysozyme and myoglobin. BSA however, was stabilised by histidine and imidazole by about 4 °C. Similarly to lysine and methylamine HCl, this is probably due to presence of multiple high affinity binding sites on the native state of BSA causing protein specific stabilisation.[19]

Although over 50 mM the data for histidine is limited, it is clear that the stabilisation of BSA by histidine plateaus and there is evidence of a small stabilisation of lysozyme. Imidazole is demonstrated to be an effective destabilising agent for all three proteins. Imidazole has a planar ring structure that is a weakly hydrated, low charge density cation and as such is not able to form strong hydrogen bonds with water.[23] Therefore it is likely to be 'pushed' onto the apolar parts of the protein due to strong water-water interactions; a hypothesis previously proposed to explain the effects of Hofmeister salts on proteins,[24] and also used to explain how guanidinium HCl destabilises proteins.[18] This results in destabilisation of the protein due to more apolar parts being exposed after unfolding. These interactions are weak and as such have very little effect on protein stability when imidazole is present at lower concentrations.

Interpretation

Direct interaction between the positively charged excipients and negatively charged side chains on the protein is evident at very low concentrations of methylamine HCl and imidazole with BSA and myoglobin, and this trend is mimicked in the amino acids lysine and histidine. Similarly BSA is stabilised in the presence of all four excipients at medium concentrations (5-25 mM), which is probably due to multiple high affinity binding sites in the native state. In a previous study guanidinium HCl, mimicking the side chain of arginine, did not induce a change in T_m that was indicative of direct interaction with the charged side chains of myoglobin or BSA and arginine's effects on T_m at low concentrations were governed by the glycine moiety.[18] This presents a level of complexity in the interaction of lysine and histidine with proteins at low concentrations that is not seen for arginine. Direct electrostatic interactions of low concentrations of inorganic salts with lysozyme have previously been shown to have measurable but unpredictable effects on thermal stability.[25] Therefore it is possible that guanidinium HCl, and therefore arginine, also directly interact with the protein surface but this has no effect on protein stability.

In the previous study arginine at low concentrations produced a protein-specific effect governed by the glycine moiety, which was attributed to weak interactions with the peptide backbone and polar side chains (figure 3). In the present study lysine and histidine at low concentrations also show more pronounced protein-specific effects than their side chains, which follow the trends of glycine.

At higher excipient concentrations low charge density ion localisation with hydrophobic surfaces can explain the destabilisation of all protein structures by imidazole, similar to guanidinium HCl. Due to the protein specific nature of the methylamine HCl trend, this is attributed to interactions with polar regions on the protein. In each case methylamine HCl, imidazole and guanidinium HCl were more destabilising than their respective amino acids lysine, histidine, and arginine due to the stabilising effect of glycine (figure 3), which was attributed to competition for water between the excipient and the unfolding protein.[25]

It has previously been suggested that leaving insoluble proteins in an imidazole-containing buffer after purification results in a more stable protein due to its positive effect on solubility.[26] Imidazole's capacity to reduce protein stability is predictable due to its ability to increase protein solubility. However, the data presented here showing destabilisation of up to 25 °C of some proteins (figure 2) means imidazole would be unsuitable as an excipient candidate. Based on this data imidazole is in fact a more potent destabiliser than guanidinium HCl, meaning it has the potential to be used in a similar way.

CONCLUSIONS

The data presented here for lysine and histidine, along with the data for arginine published previously [18] demonstrate the sometimes destabilising effects of the positively charged amino acids on a variety of model proteins. These effects on protein stability are both protein and concentration specific and as such the detrimental effects of these amino acids cannot currently be accurately predicted. It is clear from this data that both the positively charged side chains and the glycine moieties of these amino acids play an important role in the mechanisms governing their effect on protein stability. Establishment of the molecular mechanisms governing the effects of the positively charged amino acids can improve their efficacy and safety as excipients. Understanding that arginine, lysine and histidine act like a combination of their constituent groups means that there is a possibility of producing 'designer excipients' using bespoke ratios of these functional groups in solution rather than the amino acid itself in order to achieve the required characteristics of the formulation for therapeutic proteins. This work suggests a more detailed understanding of protein-excipient interactions is required than the former theories like preferential interaction and hydration that have been used to describe these interactions.[27]

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FIGURE TITLES

Figure 1. Relative change in melting temperature of 1 g/L BSA at pH 7 (red circles), 1 g/L Lysozyme at pH 7 (green triangles) and 0.5 g/L Myoglobin at pH 8 (blue squares) between the proteins in aqueous solutions and in solutions containing increasing concentrations of lysine (top panel) and methylamine hydrochloride (bottom panel)plotted on a logarithmic scale. Dotted line shows no relative change in T_m of the protein to guide the eye.

Figure 2. Relative change in melting temperature of 1 g/L BSA at pH 7 (red circles), 1 g/L Lysozyme at pH 7 (green triangles) and 0.5 g/L Myoglobin at pH 8 (blue squares) between the proteins in aqueous solutions and in solutions containing increasing concentrations of histidine (top panel) and imidazole (bottom panel). Dotted line shows no relative change in T_m of the protein to guide the eye.

Figure 3. Relative change in melting temperature of 1 g/L BSA at pH 7 (red circles), 1 g/L Lysozyme at pH 7 (green triangles) and 0.5 g/L Myoglobin at pH 8 (blue squares) between the proteins in aqueous solutions and in solutions containing increasing concentrations of glycine (adapted from a previous publication[18]) plotted on a logarithmic scale. Dotted line shows no relative change in T_m of the protein to guide the eye.

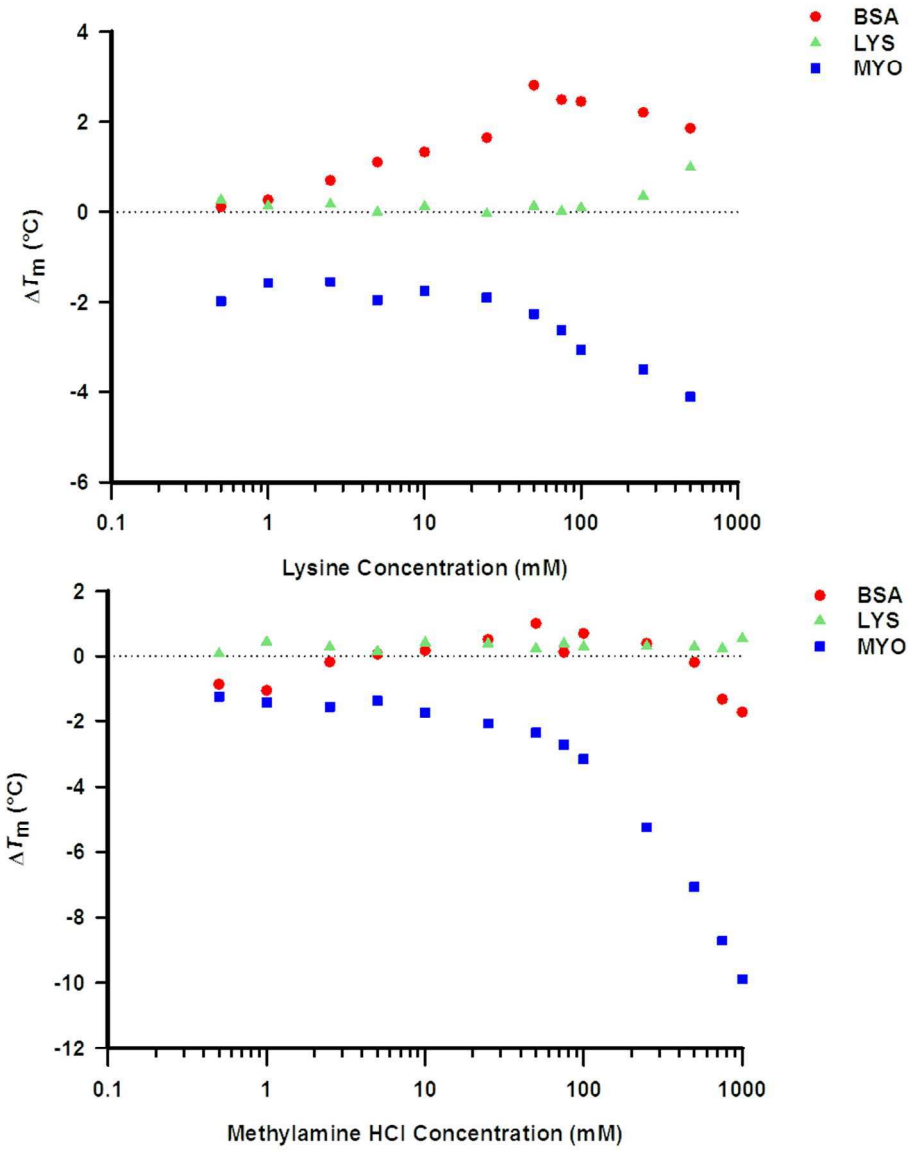

Figure 1

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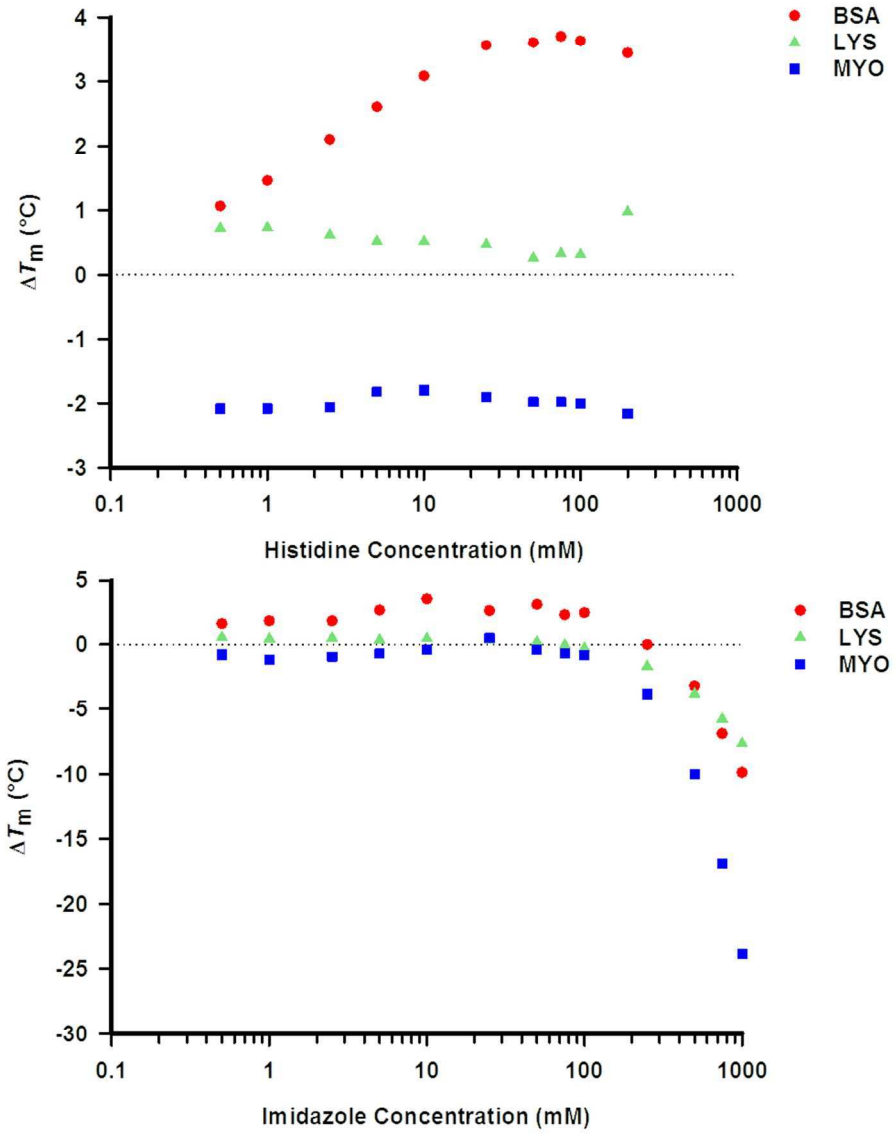

Figure 2

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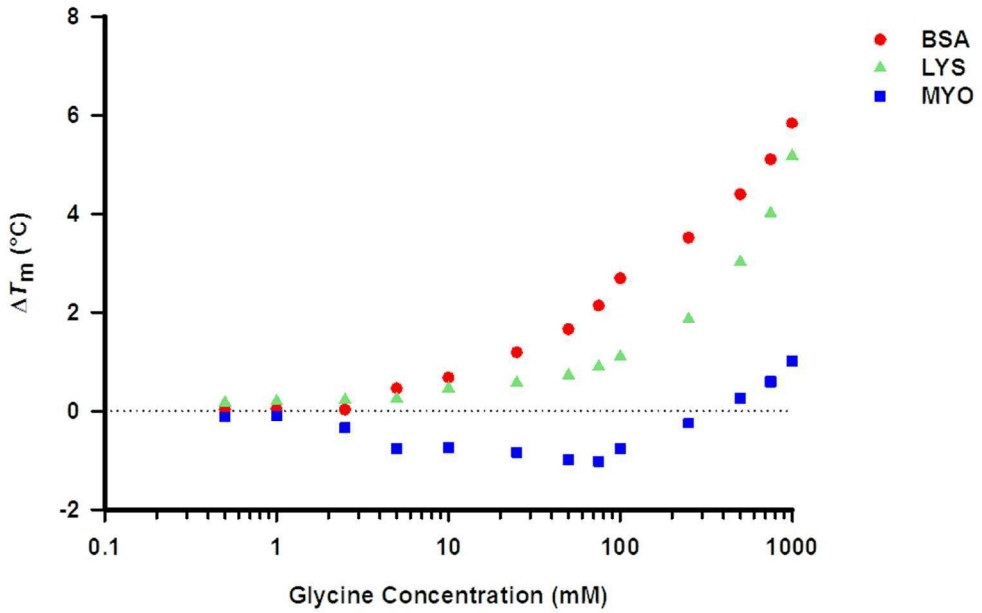
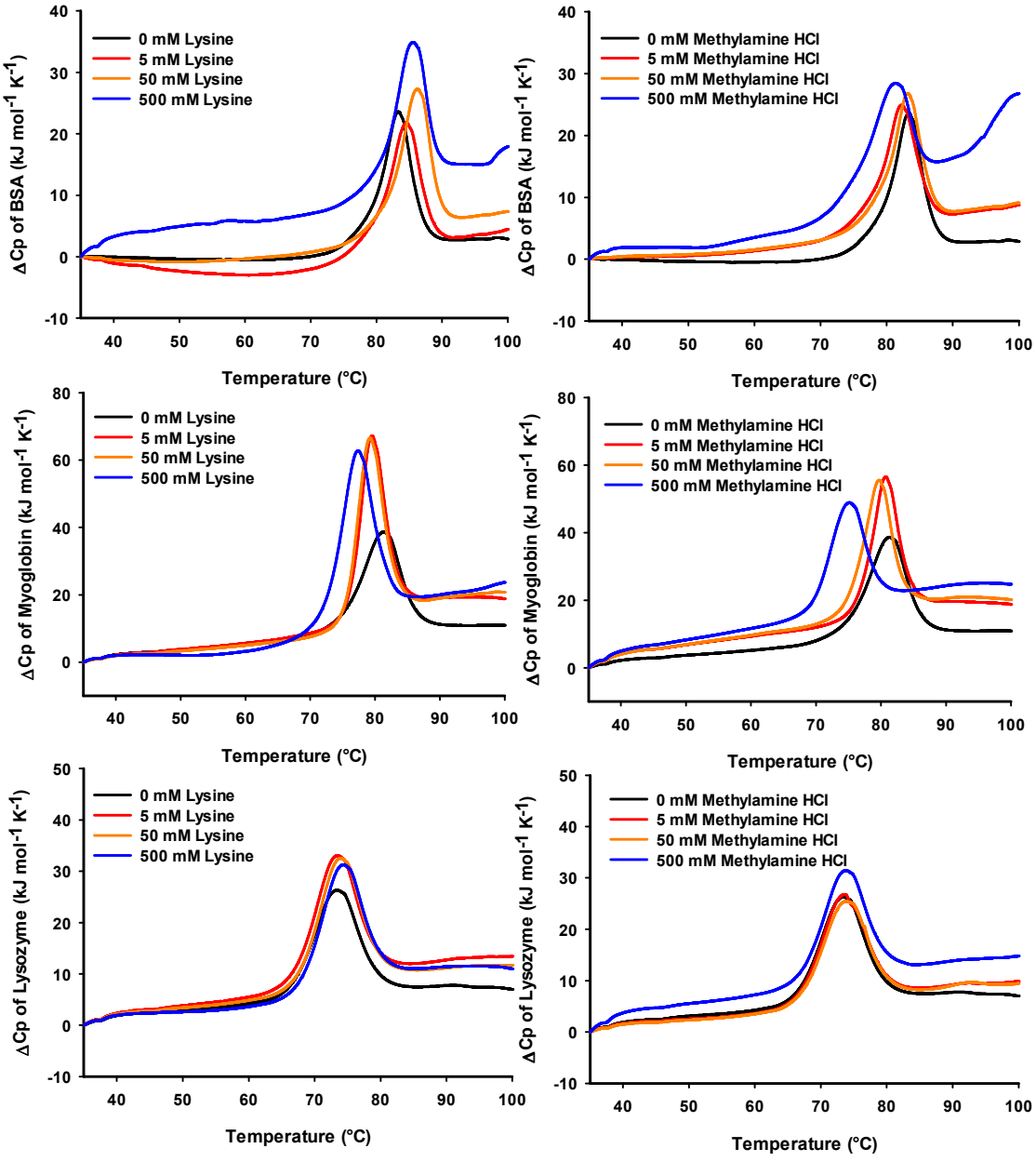


Figure 3

84x58mm (300 x 300 DPI)

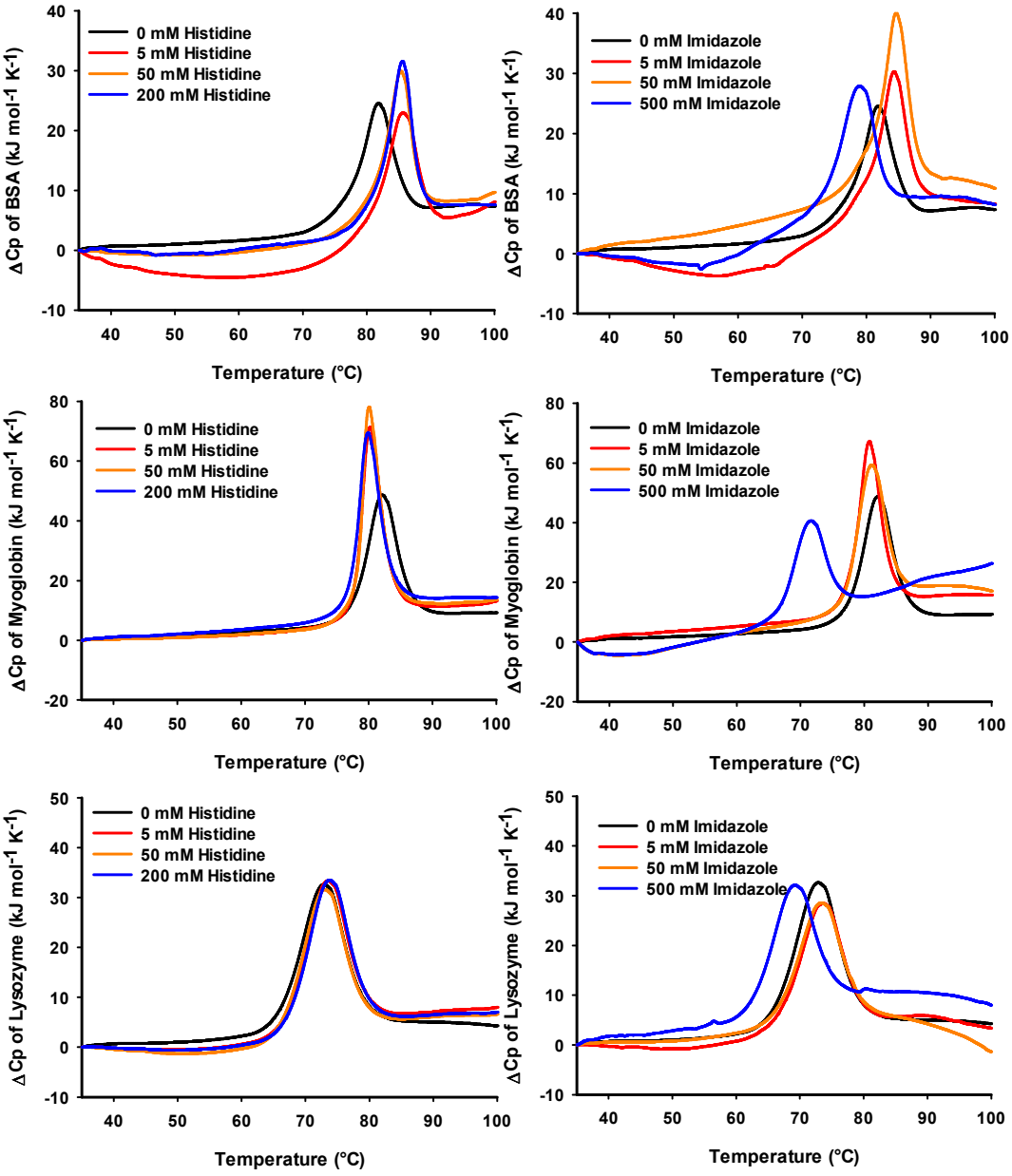
Supplementary Information for 'Control of globular protein thermal stability in aqueous formulations by the positively charged amino acid excipients'.

Lauren Platts, Samuel J Darby, Robert J Falconer*



Supplementary Figure 1. Raw DSC data showing the unfolding event of 1 g/L BSA pH 7 (top panels), 0.5 g/L Myoglobin pH 8 (middle panels) and 1 g/L Lysozyme pH 7 (bottom panels) in the presence of a low (5 mM) middle (50 mM) and high concentration (500 mM) of lysine (left panels) and

methylamine HCl (right panels). Protein scans with no cosolute present (0 mM) are plotted on each graph for comparison. T_m is calculated as the point of highest relative heat capacity.



Supplementary Figure 2. Raw DSC data showing the unfolding event of 1 g/L BSA pH 7 (top panels), 0.5 g/L Myoglobin pH 8 (middle panels) and 1 g/L Lysozyme pH 7 (bottom panels) in the presence of a low (5 mM) middle (50 mM) and high (200 mM/ 500 mM) concentration of histidine (left panels) and imidazole (right panels). Protein scans with no cosolute present (0 mM) are plotted on each graph for comparison. T_m is calculated as the point of highest relative heat capacity.