



Characterizing osmolyte chemical class hierarchies and functional group requirements for thermal stabilization of proteins

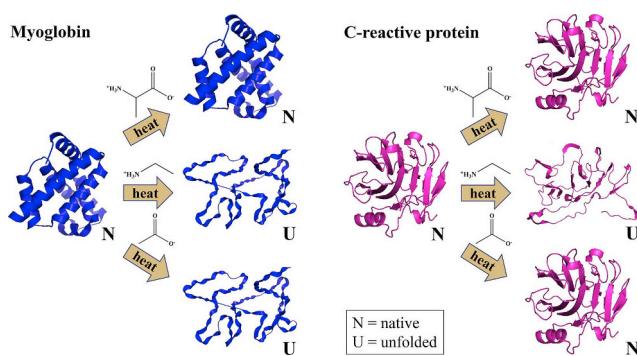
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HIGHLIGHTS

- No osmolyte class is universally superior at increasing protein thermal stability.
- Osmolyte effects on protein thermal stability are protein-specific.
- Functional group requirements for amino acid osmolytes depend on protein target.

GRAPHICAL ABSTRACT



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ABSTRACT

Osmolytes are naturally occurring organic compounds that protect cellular proteins and other macromolecules against various forms of stress including temperature extremes. While biological studies have correlated the accumulation of certain classes of osmolytes with specific forms of stress, including thermal stress, it remains unclear whether or not these observations reflect an intrinsic chemical class hierarchy amongst the osmolytes with respect to effects on protein stability. In addition, very little is known in regards to the molecular elements of the osmolytes themselves that are essential for their functions. In this study, we use differential scanning fluorimetry to quantify the thermal stabilizing effects of members from each of the three main classes of protecting osmolytes on two model protein systems, C-reactive protein and tumor necrosis factor alpha. Our data reveals the absence of a strict chemical class hierarchy amongst the osmolytes with respect to protein thermal stabilization, and indicates differential responses of these proteins to certain osmolytes. In the second part of this investigation we dissected the molecular elements of amino acid osmolytes required for thermal stabilization of myoglobin and C-reactive protein. We show that the complete amino acid zwitterion is required for thermal stabilization of myoglobin, whereas removal of the osmolyte amino group does not diminish stabilizing effects on C-reactive protein. These disparate responses of proteins to osmolytes and other small molecules are

Abbreviations: FPSA, Fractional polar surface area; CRP, C-reactive protein; TNF α , Tumor necrosis factor alpha; HEC, Hydroxyectoine; TMG, Trimethylglycine; TMAO, Trimethylamine N-oxide; GdnHCl, Guanidine hydrochloride; DSF, Differential scanning fluorimetry; T_m , Protein melting temperature; SASA, Solvent accessible surface area

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consistent with previous observations that osmolyte effects on protein stability are protein-specific. Moreover, the data reported in this study support the view that osmolyte effects cannot be fully explained by considering only the solvent accessibility of the polypeptide backbone in the native and denatured states, and corroborate the need for more complex models that take into account the entire protein fabric.

1. Introduction

Protecting osmolytes are naturally occurring, low molar mass, usually neutral, organic compounds that accumulate within the cells of organisms where they serve to maintain the structural integrity of proteins and other macromolecules against various forms of stress, including extreme pH, high pressure, osmotic stress, chemical denaturants and temperature extremes [1–4]. These compounds, which are found in all three domains of life, are typically highly soluble in water and often reach very high intracellular concentrations, in some cases reaching molar concentrations [1,5]. Evolution has selected for protecting osmolytes that impart their stabilization effects without significantly affecting protein function or cellular processes; hence these compounds are sometimes referred to as compatible solutes. There are three main chemical classes of protecting osmolytes: 1) small carbohydrates and polyols; 2) amino acids and derivatives; and 3) methylamines. In contrast to the protecting osmolytes described above, denaturing osmolytes, such as urea and guanidine, stabilize the unfolded protein state (U) relative to the native protein state (N) in the equilibrium unfolding reaction, $N \rightleftharpoons U$ [2,3].

Several different models have been proposed to explain the thermodynamics and molecular origins of osmolyte-mediated effects on protein stability, notably the transfer model and excluded volume effect [6,7]. Previous measurements of transfer free energy values for amino acids, side chains and the peptide backbone unit from water to 1 M osmolyte solutions were used in conjunction with the Tanford transfer model to describe the effects of osmolytes on protein structure in thermodynamic terms, and served as the basis for proposed explanations for the molecular origins of osmolyte effects [2,3,7–11]. These data suggested a common origin for osmolyte stabilization/destabilization effects with respect to the molecular elements of the protein critical for the response, and supported the view that osmolyte effects on protein stability are dominated by free energy contributions involving the polypeptide backbone. In contrast, the free energy contributions involving side chains were calculated to contribute relatively little toward osmolyte effects on the $N \rightleftharpoons U$ equilibrium.

Multiple studies have shown that protecting osmolytes are preferentially excluded from the protein surface whereas various denaturing osmolytes preferentially bind to the protein surface [3,6,12]. The transfer model proposes that exclusion of small protecting osmolytes is largely a consequence of their preferential hydrogen bonding interactions with water relative to the polypeptide backbone [7]. As a consequence, solvent quality is decreased by the presence of protecting osmolytes, favoring intramolecular hydrogen bonding within the polypeptide and, therefore, the folded state of the protein. According to this model, the effects of denaturing osmolytes on protein structure are a consequence of their higher binding affinities for the polypeptide backbone relative to water and the concomitant decrease in polypeptide intramolecular hydrogen bonding [6,11,13]. Another explanation for the preferential exclusion of protective osmolytes from the protein surface is the excluded volume effect, which is a steric consequence of the fact that osmolytes have a larger radius than water and are therefore preferentially excluded from the protein surface [7]. This steric exclusion is entropically unfavorable, thus favoring the more compact native state of the protein [14]. However, the fact that some denaturing osmolytes are not excluded, but instead preferentially bind to peptide groups argues for the involvement of non-steric factors in explaining osmolyte effects [7,15]. To what extent each of these effects (the excluded volume effect and the effect due to differential binding affinity

of the osmolyte for water *versus* the protein fabric) contribute to the thermodynamics of protein stabilization has yet to be fully defined.

In contrast to the body of evidence that points toward the polypeptide backbone as being the principal protein component responsible for the thermodynamics of osmolyte effects on protein stability, much less is known in regards to the principal molecular elements of the osmolytes themselves that are essential for their functions, nor in the relative effectiveness of the different osmolyte classes [16]. Biological studies have correlated the accumulation of specific classes of osmolytes, *in vivo*, to certain environmental stresses, but the functional and physicochemical bases for such correlations are unclear [3,17,18]. The three major osmolyte classes are structurally very distinct, yet empirical studies have demonstrated that members from each class can increase protein thermal stability [18–22]. However, investigations of a possible chemical class hierarchy with respect to protein thermal stability are lacking. Few reported studies of protein thermal stabilities have compared the effects of members from all three classes of protecting osmolytes [4,23–25] and most of the comparative studies that have been reported have focused on a single protein model, making it difficult to ascertain whether or not the empirical observations are broadly applicable to stabilization of globular proteins [3,16]. Similarly, very little is known in regards to the molecular elements of the osmolytes responsible for their effects on protein stability. It has been proposed that the osmolyte effect on protein stability is inversely correlated with osmolyte fractional polar surface area (FPSA) [26]; however, empirical studies of protein thermal stability have challenged this correlation [16].

Here we report our findings from a two-part investigation addressing osmolyte structural requirements for affecting protein thermal stability. In the first part of the investigation we compare the effects of members from all three major osmolyte classes on the thermal stabilities of two model proteins, human C-reactive protein (CRP) and human tumor necrosis factor alpha (TNF α). These data support the conclusion that there is no strict chemical class hierarchy amongst the osmolytes with respect to protein thermal stabilization effects. In addition, these data are consistent with previous observations that the relative effects of osmolytes on protein thermal stability are protein-specific. Moreover, these data support the view that osmolyte effects on protein stability are highly nuanced and cannot be fully explained by considering only the solvent accessibility of the polypeptide backbone in the native and denatured conformations. In the second part of the investigation, we dissect the structural requirements of amino acid osmolytes for imparting protein thermal stability to CRP and human myoglobin. The results from this part of the study reveal that the functional groups on amino acid osmolytes required to increase protein thermal stability differ between CRP and myoglobin. These data support the observation from the first part of the study that solute effects (in this case, non-osmolytes) on protein thermal stability are highly protein-specific. Taken together, these findings have potentially important implications in the development of formulations for broad-spectrum protein stabilization in biospecimens for medical diagnostics and for the design of formulations to stabilize proteins for biotechnological or therapeutic applications.

2. Materials and methods

2.1. Materials

Recombinant human tumor necrosis factor alpha (TNF α) was purchased from BioLegend. Specifically, the soluble form of human TNF α

(corresponding to amino acids 77–233 of the full-length pro-TNF protein) was used in this study. Recombinant human C-reactive protein (CRP) and myoglobin from human heart were purchased from Lee BioSolutions. All osmolytes and other compounds were reagent grade or higher. Stock solutions of fructose, glucose, lactose, sucrose, trehalose, L-alanine, glycine, trimethylglycine (TMG), ectoine, hydroxyectoine (HEC), trimethylamine N-oxide (TMAO), urea, guanidine HCl (GdnHCl), β -alanine, sodium acetate, sodium propionate, ethylamine, propylamine, and ethanolamine were prepared in deionized water and adjusted to pH 7.5 \pm 0.5 with sodium hydroxide or hydrochloric acid, as necessary.

2.2. Differential scanning fluorimetry

Thermal denaturation studies were carried out in a StepOne Real-Time PCR System equipped with a 48-well block (Applied Biosystems, USA). Each test sample contained 1.5 μ g of protein and 50 mM potassium phosphate, pH 7.5 at a final volume of 20 μ L. Test samples for thermal denaturation analysis of CRP and TNF α included a final concentration of 1 \times Protein Thermal Shift Dye (Applied Biosystems, USA). Myoglobin samples included a final concentration of 1 \times Sypro Orange (Sigma-Aldrich) in lieu of 1 \times Protein Thermal Shift Dye because the latter dye resulted in a weak fluorescence signal. Osmolytes were added to the reaction mixtures at the final concentrations indicated in the results section. Potential effects of osmolytes on reaction pH were tested by measuring the reaction mixture pH at the highest osmolyte concentration investigated. For all osmolytes in the study, the final pH of the reaction mixture was 7.5 \pm 0.5. Data from the first part of the study (comparative analysis of the effects of thirteen osmolytes from different classes on the thermal stabilities of CRP and TNF α) were fit to the two-state protein unfolding model described by Ramsay and Eftink [27] that takes into account the pre-transition and post-transition baseline data in the fit to derive protein melting temperature (T_m). Data from the second part of the study (amino acid osmolyte structural requirements for protein-stabilizing activity) were fit to a Boltzmann function according to the procedure described by Wright *et al* [28] to determine protein T_m . This model also assumes a two-state protein unfolding model. (Analysis of the data from the first part of the study revealed that the different fitting models resulted in only small differences in T_m and provided the same rank order of osmolyte effectiveness; therefore we used the simpler Boltzmann function, which has fewer floating parameters, to analyze the data for part two of the investigation). Protein T_m values are reported as the average of three replicate measurements \pm standard deviation (using the STDEV-S function).

2.3. Determination of protein solvent accessible surface area

For each protein in this study, Surface Racer [29] was used to calculate the fractional solvent accessible surface area (SASA) of each of the following component groups of the native protein: polar, non-polar, backbone, polar side-chain, non-polar side chain, positive charge and negative charge groups. SASA of the native protein structures was also calculated using GETAREA [30] which provided total SASA, backbone SASA, sidechain SASA, polar SASA, and non-polar SASA for each amino acid. A probe radius of 1.4 Å was used for these calculations. The following atomic coordinate files were used for CRP, TNF α and myoglobin, respectively: 1gnh, 1tnf and 3rgk. SASA for the unfolded proteins was calculated using ProtSA [31,32]. The mean total SASA, mean backbone SASA, mean sidechain SASA, mean polar SASA, and mean non-polar SASA was calculated for each amino acid from 2000 predicted unfolded conformations.

2.4. Determination of osmolyte fractional polar surface area

The osmolyte molecules were built in Spartan'14 (Wavefunction, Inc.) and their aqueous geometry was determined using the *Equilibrium*

Geometry option. The minimization was based on HF calculations using the 6-31G* basis and water as the solvent. After the geometry optimization Spartan then generated the total surface area and polar surface area, according to the CPK model. The ratio of these two quantities determined the FPSA.

2.5. m-value calculations

Predicted m-values were calculated using the method described by Auton and Bolen [10]. In brief, this method allows for the calculation of solvent-dependent protein unfolding free energy changes (m-values) using transfer free energies of amino acid side chains and backbone units from water to various solvents in combination with Tanford's transfer model. The calculated m-value (m_{calc}) for a protein with a specific osmolyte was determined using equation [1]:

$$m_{\text{calc}} = \sum_{i=\text{amino acid type}} n_i \Delta g_{tr,i}^{\text{sc}} \alpha_i^{\text{sc}} + \Delta g_{tr}^{\text{bb}} \sum_{i=\text{amino acid type}} n_i \alpha_i^{\text{bb}} \quad [1]$$

In this equation, n_i is the number of amino acids of type i in the protein; $\Delta g_{tr,i}^{\text{sc}}$ and $\Delta g_{tr}^{\text{bb}}$ are the transfer free energies from water to 1 M osmolyte for the sidechain and peptide backbone unit, respectively; α_i^{sc} and α_i^{bb} are the fractional change in solvent accessibility of amino acid i going from the native protein conformation to denatured conformation for the sidechain and peptide backbone unit, respectively (see [10] for a more thorough description). The sidechain and backbone solvent accessible surface areas for the native and denatured conformations were determined using GETAREA and ProtSA, respectively, as described above. Amino acid sidechain and peptide backbone unit transfer free energies for each amino acid into osmolyte solutions were obtained from [10], as were the sidechain and backbone unit tripeptide solvent accessibility values for each amino acid (which were used in the calculations of α_i^{sc} and α_i^{bb}). m-values were calculated for each osmolyte involved in this study for which transfer free energy values were available in the literature (TMAO, sucrose and TMG).

3. Results

3.1. Osmolyte effects on protein thermal stability: protein-specificity and chemical class hierarchies

In the first part of this investigation we measured the effects of thirteen natural osmolytes on the thermal stabilities of two proteins, human C-reactive protein (CRP) and human tumor necrosis factor alpha (TNF α). This part of the study was focused on addressing the primary question of whether or not there exists a hierarchy amongst the major osmolyte classes with respect to their effects on protein thermal stability. We also tested whether the relative effects of osmolytes on the thermal stabilities of CRP and TNF α are the same, or whether osmolyte effects are protein-specific as has been reported in other studies *e.g.* [22]. In addition, we tested whether or not our empirical stabilization data could be explained based on differences in osmolyte molar mass, or whether these data matched predictions based on the transfer model.

Included in this study were eleven compounds generally categorized as protecting osmolytes. Representatives from each of the three main osmolyte chemical classes were selected for investigation. The carbohydrates included two monosaccharides (fructose and glucose) and three disaccharides (sucrose, lactose and trehalose). Two proteogenic amino acids were included (glycine and L-alanine), as well as two amino acid derivatives (ectoine and hydroxyectoine (HEC)). The methylamines selected for investigation were trimethylglycine (TMG) and trimethylamine N-oxide (TMAO). Also included in this investigation were two protein denaturing osmolytes, urea and guanidine hydrochloride (GdnHCl). The structures of the osmolytes analyzed in part one of this study are shown in Fig. S1. CRP and TNF α were selected as model proteins for three primary reasons: 1) they are structurally similar; 2) both proteins display unfolding behavior consistent with a

two-state protein folding/unfolding transition, thus simplifying data analysis and interpretation; and 3) they are diagnostically important proteins [33–35] and are therefore relevant targets for improved chemistries to maintain analyte integrity during the preanalytical phase of biospecimen storage and transport. CRP and TNF α are similar in amino acid composition (Tables S1, S2) and both proteins are homo-multimeric, globular proteins comprised of relatively small subunits (23.0 kDa and 17.4 kDa, respectively). CRP and TNF α are both primarily β -strand in secondary structure composition (46% and 41%, respectively) with little α -helical content (7% and 1%, respectively) [36,37]. In addition, CRP and TNF α native structures are highly similar in the sidechain and polypeptide backbone contributions from each class of amino acids to the solvent accessible surface area (SASA) of the native protein (Tables S3, S4), as well as in the fractional accessible surface areas of their total polar, total non-polar and total backbone components (Table S5). Structurally similar protein models were selected based on the premise that if osmolytes were to possess a general (*i.e.* not protein-specific) hierarchy in their capacities to affect protein thermal stability then this hierarchy would be expected to be especially apparent for structurally similar proteins. Conversely, if the rankings of osmolyte activities were found to be different for structurally similar proteins then this would suggest that the relative effects of osmolytes on protein thermal stability are highly protein-specific. It is worth noting that whereas these proteins are similar in terms of overall amino acid composition and native state SASA composition, these are not homologous proteins, as indicated by amino acid sequence alignments (Table S6, Fig. S2).

Protein stability as a function of osmolyte concentration was measured by differential scanning fluorimetry (DSF). Thermal unfolding data were fit to a model describing a two-state unfolding transition allowing determination of protein melting temperature (T_m). The goodness of fit for CRP and TNF α data to this model supported the assumption of a two-state transition for each of these proteins under all conditions tested (Fig. 1). CRP T_m was observed to increase with increasing concentration of each of the eleven osmolytes generally classified as protein-stabilizing. For six of these osmolytes (fructose, glucose, lactose, trehalose, glycine and HEC), T_m increased linearly up to the highest concentration tested (Fig. 2a,b). Linearity was also observed for the protein denaturing osmolytes, urea and GdnHCl, albeit with T_m values *decreasing* with increasing osmolyte concentration, as expected (Fig. 2c). For the other five osmolytes generally classified as protecting, the relationship between CRP T_m and osmolyte concentration became non-linear above some threshold concentration, either plateauing at sufficiently high concentrations (sucrose, L-alanine, ectoine and TMAO) or displaying a parabolic relationship (TMG) (Fig. S3). Such non-linearity in the response of protein stability to osmolyte concentration has

been reported previously *e.g.* [22,38].

TNF α T_m was observed to increase with increasing concentrations of ten of the eleven osmolytes generally classified as protein-stabilizing, the exception being ectoine which had no significant effect on thermal stability at concentrations up to 1.0 M (Fig. 3). The correlation of TNF α T_m with osmolyte concentration was linear up to the maximum concentration measured for 11 of the 13 osmolytes tested (including the denaturing osmolytes, urea and GdnHCl) (Fig. 3), but was observed to plateau at L-alanine concentrations above 0.4 M (Fig. S4). Ectoine, generally considered to be a protein-stabilizing osmolyte, was observed to lower TNF α T_m at a sufficiently high concentration (2.0 M) (Fig. S4). The dramatically different effects of ectoine on the thermal stabilities of CRP and TNF α are consistent with previous reports that osmolyte effects can be highly protein-specific *e.g.* [4].

Osmolyte effects on protein thermal stability were quantified by two metrics: molar m-value and mass per volume m-value. Molar m-value was defined as the slope of the linear fit of T_m versus molar osmolyte concentration [24]; for osmolytes that displayed non-linearity at high concentrations, only data points in the lower concentration linear range were included for m-value determination (Figs. 2, 3). Because molar m-value excludes effects due to differences in osmolyte solubility or non-linearity at higher concentrations [22], this metric represents a quantifiable means to compare intrinsic osmolyte activities with regards to protein thermal stabilization.

Molar m-values for CRP and TNF α thermal stabilization are summarized in Table 1 and Fig. S5. The rank order of osmolyte effectiveness based on this metric clearly demonstrated, for each protein model, a lack of an osmolyte activity hierarchy based strictly on chemical class. In addition, the data indicated that the relative effects of osmolytes on protein thermal stability are highly protein specific. For CRP, the rank order of protecting osmolyte effectiveness based on molar m-values was (from most active to least): ectoine > lactose > L-alanine > trehalose > TMAO > glucose > HEC > sucrose > fructose > TMG > glycine. For TNF α , the protecting osmolyte rank order was: L-alanine > lactose > trehalose > glycine > sucrose > TMAO > HEC >

TMG > fructose > glucose > ectoine. For both CRP and TNF α an amino acid (or derivative) was the most active thermal stabilizer based on molar m-values; however, the optimal compound differed for the two proteins (ectoine for CRP and L-alanine for TNF α) and amino acids, as a group, were *not* more effective thermal stabilizers than the other chemical classes. The most pronounced difference in the osmolyte responses between the two proteins was that ectoine, the most active stabilizer for CRP, was inactive with respect to TNF α thermal stabilization at lower concentrations (and indeed, de-stabilizing at high concentrations) (Figs. S4, S5). Conversely, L-alanine had only a modest effect on CRP thermal stability (relative to the other osmolytes)

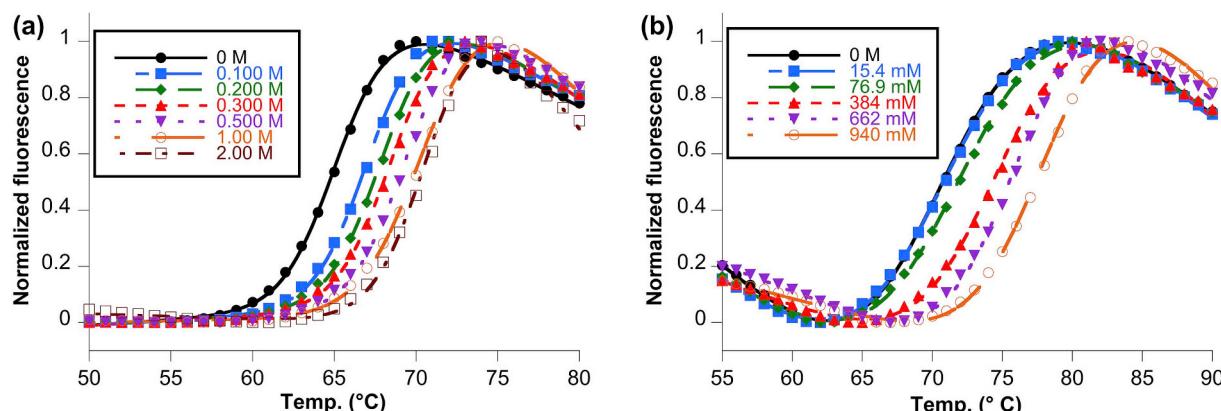


Fig. 1. Differential scanning fluorimetry (DSF) analysis of CRP in the presence of increasing concentrations of ectoine (a) and TNF α in the presence of increasing concentrations of L-alanine (b). Fluorescence values were normalized for presentation purposes. Unadjusted fluorescence data were fit to a model describing a two-state protein folding/unfolding transition to determine protein melting temperature (T_m) [27].

whereas it was the most effective stabilizer for TNF α (Fig. S5).

Next we compared the experimentally determined osmolyte rank orders for CRP and TNF α stabilization to the rank order of osmolytes that would be predicted if the SASA of the polypeptide backbone alone (and not the SASA of sidechains) was the only contributing factor of the

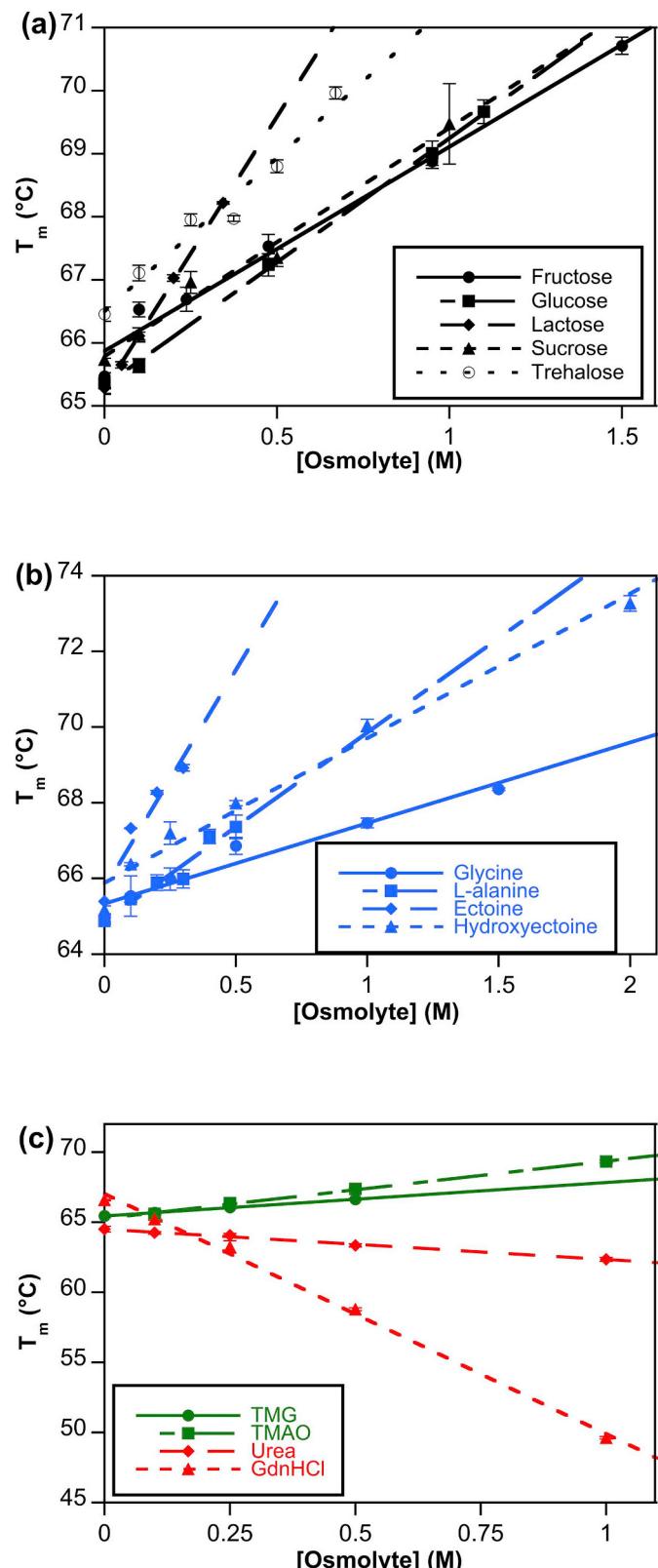


Fig. 2. Linear response of CRP T_m as a function of osmolyte concentration. CRP T_m values were plotted against carbohydrate (a), amino acid (b), methylamine and denaturant (c) molar concentrations. The concentration range measured for each osmolyte was based upon compound solubility limits except for ectoine, hydroxyectoine (HEC), trimethylglycine (TMG) and trimethylamine N-oxide (TMAO) which were measured up to 2.0 M final concentrations, and urea and guanidine HCl (GdnHCl) which were measured up to 1.0 M final concentrations. The molar m-value of each osmolyte was determined by linear regression. Error bars represent the standard deviation of three replicates. For sucrose, L-alanine, ectoine, TMG and TMAO, data points outside the linear range were excluded from the linear fit and are not shown (see Fig. S3 for complete data sets). Linear fits are extrapolated to graph boundaries for clarity.

protein determining osmolyte effectiveness. Based on transfer free energy values for the polypeptide backbone unit into osmolyte solutions, the predicted rank order of osmolyte effectiveness would be: TMAO > TMG > sucrose [9,10,26]; this does not match the experimentally determined rank order of either protein model. This finding, in conjunction with the observation that the fractional change in the SASA of the polypeptide backbone in going from the native conformation to the denatured state is very similar for CRP and TNF α (compare Tables S7, S8), argues that osmolyte effects cannot be explained by considering only interactions with the backbone and corroborates the need for models that include side chain contributions in calculating osmolyte effects (e.g transfer model [10]).

We then compared the experimentally determined osmolyte rank orders for CRP and TNF α stabilization to the rank orders predicted by the Tanford transfer model using the method described by Auton and Bolen [9,10]. In brief, this method allows for the calculation of solvent-dependent protein unfolding free energy changes (m-values) using transfer free energies of amino acid side chains and backbone units from water to various solvents in combination with Tanford's transfer model. The predicted m-values based on the transfer model, as shown in Table S9, are remarkably close for CRP and TNF α and are consistent with the similarity of these two proteins in terms of amino acid composition, native state SASA, and fractional change in SASA in going from the native to denatured conformations (as described above). For both protein models, the predicted rank order of protecting osmolyte effectiveness was TMAO > sucrose > TMG (Table S9). Though the number of osmolytes for which these calculations could be performed is small, this rank order does match the experimental rank order for CRP, thus supporting the validity of the transfer model. For TNF α , however, the predicted and experimental rank orders do not match (experimental rank order for TNF α was sucrose > TMAO > TMG). (It is worth noting that the units for the calculated m-values using the transfer model differ from the units for our experimentally determined m-values, so our comparisons are based solely on osmolyte rank orders.) With this limited data set it is impossible to draw strong conclusions about the broad applicability of the Tanford transfer model as a mechanistic explanation for osmolyte-mediated stabilization of proteins; however, these data do suggest the possibility that TNF α may have unique properties that account for its differential response to osmolytes (see Discussion).

Next, we investigated the possibility that the differences in the empirical osmolyte molar m-values (either within or between chemical classes) were a consequence of differences in molar mass. We hypothesized that normalizing osmolyte activity to osmolyte molar mass may reveal similar thermal stabilization activities within a chemical class, or reveal intrinsic similarities/differences between osmolyte chemical classes. To this end, we reanalyzed the DSF data to calculate mass per volume m-values, as defined as the slope of the linear fit of T_m versus g/L osmolyte concentration (for osmolytes that displayed non-linearity at high concentrations, only data points in the linear range were included for m-value determination, as described above for molar m-values). Linear fits for mass per volume m-value calculations for CRP and TNF α are shown in Figs. S6 and S7, respectively, and summarized in Table 1 and Fig. S8. If osmolyte effects on thermal stability were

dominated by the size (or molar mass) of the compound then similar mass per volume m-values would be expected; however, for each model protein system, a comparison of mass per volume m-values for osmolytes from different chemical classes revealed significantly different values. Consistent with this observation, no correlation was observed

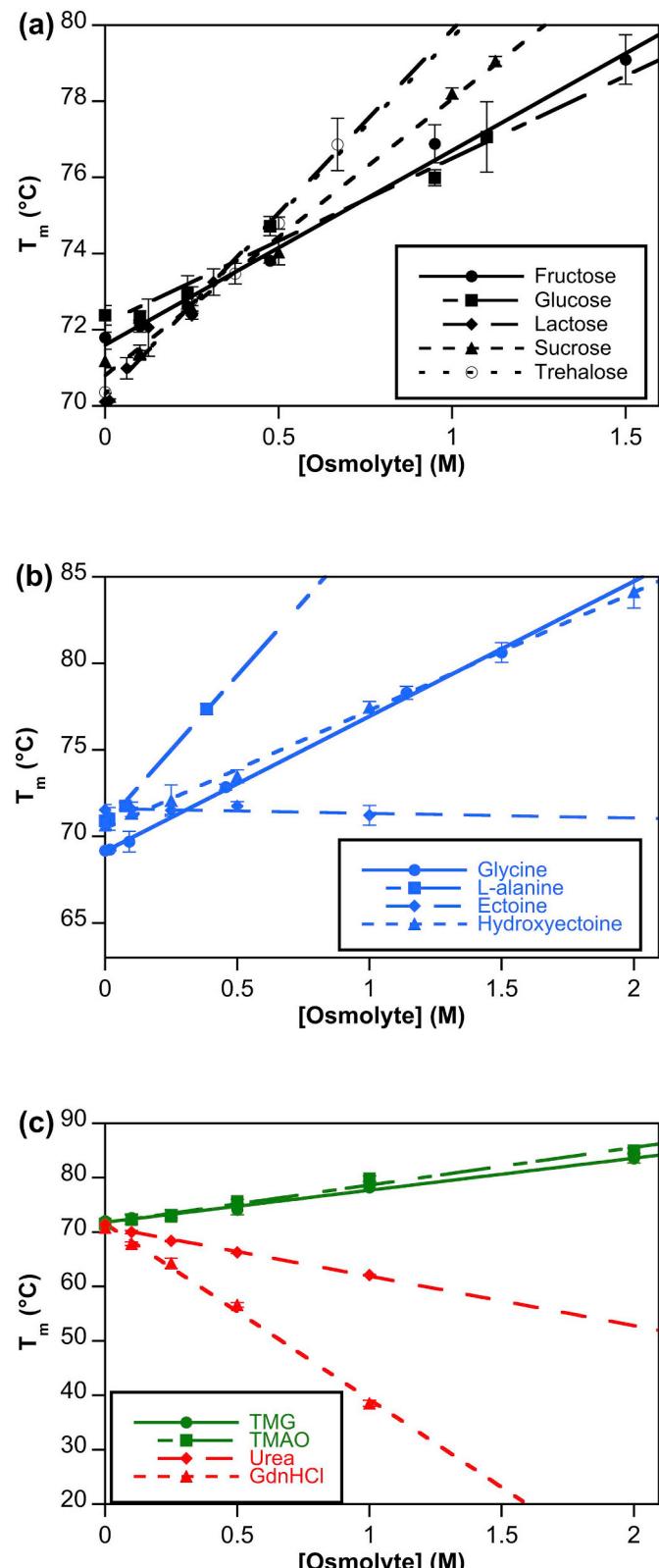


Fig. 3. Linear response of TNF α T_m as a function of osmolyte concentration. TNF α T_m values were plotted against carbohydrate (a), amino acid (b), methylamine and denaturant (c) molar concentrations. The concentration range measured for each osmolyte was based upon compound solubility limits except for ectoine, hydroxyectoine (HEC), trimethylglycine (TMG) and trimethylamine N-oxide (TMAO) which were measured up to 2.0 M final concentrations, and urea and guanidine HCl (GdnHCl) which were measured up to 1.0 M final concentrations. The molar m-value of each osmolyte was determined by linear regression. Error bars represent the standard deviation of three replicates. For L-alanine and ectoine, data points outside the linear range were excluded from the linear fit and are not shown (see Fig. S4 for complete data sets). Linear fits are extrapolated to graph boundaries for clarity.

Table 1

Summary of osmolyte effects on the thermal stabilities of CRP and TNF α .

Osmolyte	CRP m-value, ($^{\circ}\text{C}$ M) ^a	CRP m-value, ($^{\circ}\text{C L/g}$) ^b	TNF α m-value, ($^{\circ}\text{C}$ M) ^a	TNF α m-value, ($^{\circ}\text{C L/g}$) ^b
Fructose	3.2	1.8	5.1	2.8
Glucose	3.9	2.2	4.3	2.4
Lactose	8.7	2.5	9.6	2.8
Sucrose	3.6	1.1	7.3	2.1
Trehalose	4.9	1.4	9.4	2.7
Glycine	2.1	2.8	7.8	10.4
L-alanine	5.0	5.6	17.2	19.3
Ectoine	11.5	8.1	-0.3	-0.2
Hydroxyectoine	3.8	2.4	6.8	4.3
Trimethylglycine	2.4	2.0	5.9	5.0
TMAO	4.1	5.4	6.9	9.2
Urea	-2.2	-3.6	-9.1	-15.1
Guanidine HCl	-17.1	-17.9	-32.3	-33.8

^aMolar m-value, calculated from the slope of the plot of T_m versus molar osmolyte concentration, as shown in Figs. 2 and 3. ^bMass/Volume m-value, calculated from the slope of the plot of T_m versus g/L osmolyte concentration, as shown in Fig. S6 and Fig. S7.

between molar m-values and osmolyte total accessible surface area for either protein system (data not shown).

We also analyzed mass per volume m-values to determine if differences in osmolyte activities *within* a chemical class could be explained by differences in molar mass. For each protein system, osmolytes within each of the amino acid, methylamine and denaturant classes revealed significantly different mass per volume m-values, suggesting that their effects on stabilization could not be explained solely by considerations of molar mass (Table 1 and Fig. S8). However, when normalized for differences in molar mass, each of the carbohydrates were very similar with respect to TNF α thermal stabilization. But this trend was not observed for CRP; in this case, the mass per volume m-values of the disaccharides sucrose and trehalose were significantly lower than the other carbohydrates. These findings from mass-per-volume m-value comparisons agree with previous studies that indicate that additional factors besides osmolyte molar mass determine effects on protein stabilization.

Taken together, the DSF data described above are consistent with previous reports that osmolyte activity with respect to protein thermal stabilization is highly protein-specific, as indicated by the differences in the rank order of osmolyte effectiveness observed for CRP and TNF α . Importantly, these data also suggest that no one osmolyte class (as a whole) is inherently the most effective at increasing protein thermal stability for all protein targets.

3.2. Osmolyte structural requirements for protein-stabilizing activity

Previous efforts to define the physicochemical properties responsible for osmolyte-induced effects on protein stability have shown that transfer free energy values for the polypeptide backbone into

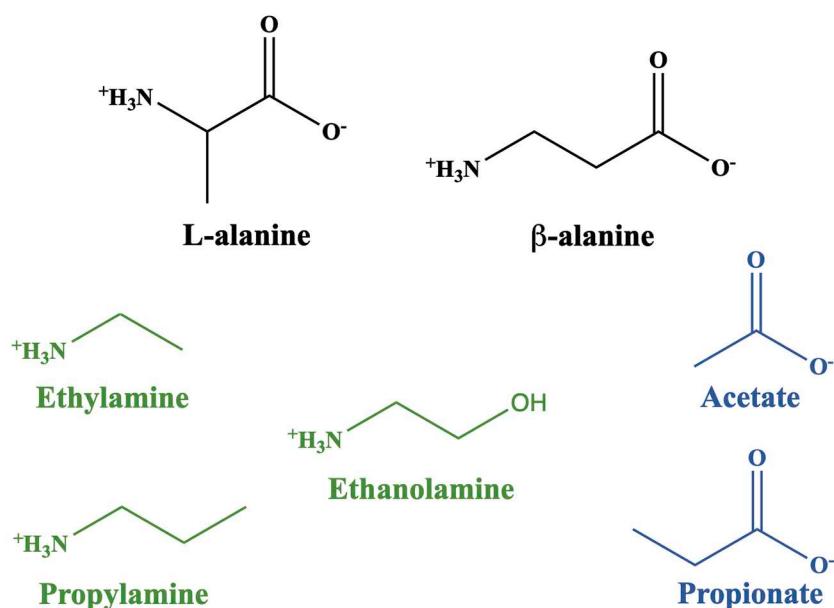


Fig. 4. Structures of the seven compounds tested to determine functional group requirements for amino acid-induced protein thermal stabilization.

osmolyte solutions inversely correlate with osmolyte fractional polar surface area (FPSA) [26]. In conjunction with the transfer model and the conclusion that osmolyte effects are dominated by interactions with the backbone, this has led to the prediction that protein stabilizing effects should also inversely correlate with osmolyte FPSA. However, our empirical thermal stability data for CRP and TNF α indicated no correlation between osmolyte FPSA and molar m-values for either model protein system (Fig. S9). To gain better insight into the physicochemical properties of osmolytes responsible for their thermal stabilizing effects we focused on the functional group requirements of amino acid osmolytes. To this end, we conducted DSF analyses of protein thermal stability as a function of seven compounds (Fig. 4) [16]. Two amino acid osmolytes were included in this analysis, L-alanine and β -alanine. The other five compounds represented a structural dissection to identify amino acid functional group contributions to protein thermal stability.

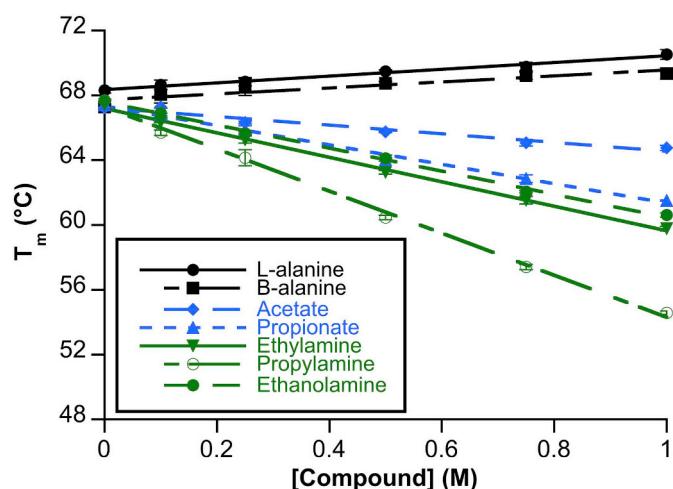


Fig. 5. Amino acid functional group effects on myoglobin thermal stability. Myoglobin T_m was measured as a function of the concentration of the amino acids L-alanine and β -alanine, and compounds lacking the amino group (acetate and propionate) or lacking the carboxylate group (ethylamine and propylamine). Ethanolamine was tested to assess the effect of substituting the carboxylate group with a hydroxyl group. The molar m-value for each osmolyte was determined by linear regression. Error bars represent the standard deviation of three replicates.

Ethylamine and propylamine were analyzed to ascertain the thermal stabilizing properties of compounds lacking the carboxylate present in amino acids. Acetate and propionate were analyzed to determine the thermal stabilizing properties of compounds lacking the amino group. And ethanolamine was included in this study to determine the effect of replacing the amino acid carboxylate group with a hydroxyl group.

The effects of this series of compounds on protein thermal stability were measured for two protein model systems: myoglobin and CRP. These proteins differ significantly in secondary structure composition (the helical/ β -strand compositions of myoglobin and CRP are 77%/0% and 7%/46%, respectively), but (like the proteins in part I of this study) they are similar in fractional accessible surface area composition in their native conformations (Table S1). CRP and myoglobin are also very similar in the fractional change in SASA for the polypeptide backbone in going from the native to denatured conformations (compare Tables S7 and S10). We hypothesized that if small molecule effects on protein stability are driven primarily by interactions with the accessible polypeptide backbone in the native and denatured conformations, in a sequence-independent manner, then these two proteins should respond similarly to this series of compounds.

The amino acids L-alanine and β -alanine functioned as protective osmolytes of myoglobin with similar molar m-values, as determined by DSF analysis (Fig. 5 and Table 2). Removal or substitution of either the carboxylate or amino group effectively abrogated all stabilization activity and resulted in compounds that thermally destabilized myoglobin. Removal of the amino acid carboxylate group had a greater

Table 2
Compound effects on myoglobin thermal stability.

Osmolyte	m-value ($^{\circ}\text{C}/\text{M}$) ^a
L-alanine	2.1
β -alanine	1.8
Acetate	-2.7
Propionate	-6.0
Ethylamine	-7.6
Propylamine	-13.0
Ethanolamine	-7.1

^a Molar m-value, calculated from the slope of the plot of T_m versus molar test compound concentration, as shown in Fig. 5.

destabilizing effect compared to removal of the amino group as indicated by the more negative molar m-values for ethylamine/propylamine versus acetate/propionate (Table 2). Comparing these data to ethanolamine indicated that substitution of the amino acid carboxylate with a hydroxyl group did not restore the stabilization functionality of the compound, indicating that the complete, zwitterionic amino acid structure is required for thermal stabilization of myoglobin.

Significantly different results were observed for this series of compounds with respect to CRP thermal stability. L-alanine and β -alanine functioned as thermal stabilizers of CRP (Fig. 6a). However, in stark contrast to the destabilizing effects of acetate and propionate on myoglobin thermal stability, both of these compounds functioned to increase the thermal stability of CRP (compare Fig. 5 and Fig. 6a). Removal of the amino acid carboxylate group also had a significantly different effect on CRP thermal stability than was observed for myoglobin. At low concentrations ethylamine, propylamine and ethanolamine increased CRP stability, but at higher concentrations they functioned as destabilizers (Fig. 6b). In the case of CRP, the destabilizing effect of carboxylate removal was partially restored by substitution with a hydroxyl group (compare ethanolamine to ethylamine).

Unlike the results for myoglobin, the data for CRP indicated that each of the seven compounds involved in this analysis had thermal stabilization activity, at least over some limited concentration range. In order to quantify the stabilizing effects of these compounds on CRP, DSF analysis was conducted over more narrow compound concentrations ranges and molar m-values were calculated from the linear response regions (Fig. 7). The broad range and narrow range titration data from Figs. 6 and 7 are summarized in Table 3. Surprisingly, the compounds lacking the amino group (acetate and propionate) proved to be more effective thermal stabilizers of CRP than both of the amino acid osmolytes based on comparisons of molar m-values. The high stabilization activity of ethanolamine at low compound concentrations, as reflected in its molar m-value, is remarkable. It should be noted, however, that for the compounds lacking the carboxylate group (ethanolamine, propylamine, and ethylamine) these molar m-values were calculated over very low compound concentrations; at higher concentrations, each of these compounds were significantly less effective in stabilizing CRP than the complete amino acids or the compounds lacking the amino groups.

In summary, the data from the structural dissection of amino acid osmolytes indicated that the complete, zwitterionic amino acids functioned as thermal stabilizers of both myoglobin and CRP; however, removal of either the carboxylate or amino group resulted in dramatically different effects on the thermal stabilities of the two model proteins. Most striking were the thermally destabilizing effects of acetate and propionate on myoglobin versus their stabilizing effects on CRP.

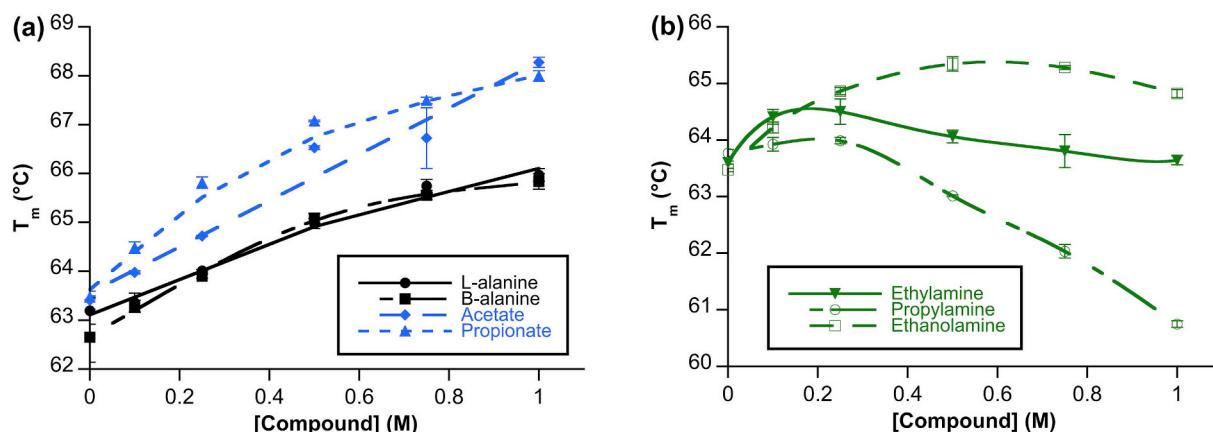


Fig. 6. Amino acid functional group effects on CRP thermal stability. CRP T_m was measured over a broad concentration range of the amino acids L-alanine and β -alanine, and compounds lacking the amino group (acetate and propionate) (a) or lacking the carboxylate group (ethylamine, propylamine and ethanolamine) (b). Error bars represent the standard deviation of three replicates.

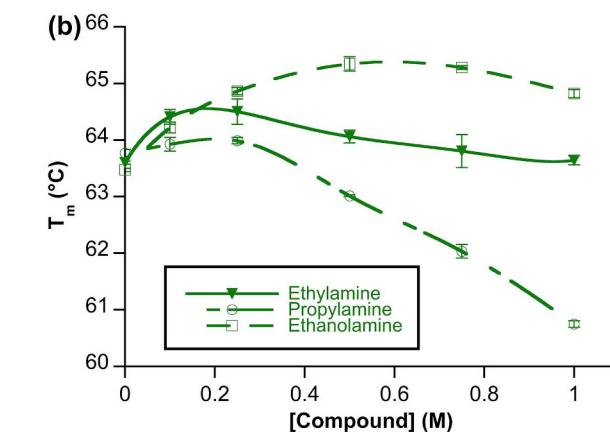
These results are consistent with the conclusion from the first part of this investigation, namely that solute effects on protein stability are highly protein-specific and suggest that the functional group requirements for osmolyte-mediated effects on protein stability may also be highly protein-dependent.

4. Discussion

The full realization of the potential of osmolytes to stabilize proteins in medical and biotechnological applications requires a thorough understanding of the mechanism of osmolyte-induced protein stabilization and the physicochemical properties of both the protein target and osmolyte responsible for the effect. The data presented in this study support two major conclusions: 1) There is no strict chemical class hierarchy amongst osmolytes with respect to their effects on protein thermal stability; and 2) The effects of osmolytes on protein thermal stability are highly protein specific. These data also support previous observations that osmolyte-mediated effects on protein thermal stability cannot be fully explained by considerations of polypeptide backbone solvent accessibility alone.

Biological studies have suggested that certain classes of osmolytes may be more optimized to counteract specific forms of stress. For example, studies of organisms that accumulate high concentrations of urea have indicated the concomitant accumulation of specific methylamines, suggesting that members from this class of osmolytes are optimized for counteracting chemical denaturants [3,17,39–42]. Similar analyses have suggested that evolution has preferentially selected polyols and carbohydrates to counteract temperature stresses, whereas amino acids have been preferentially selected to oppose osmotic stress [3,4,17,40,43]. However, it is not clear if these *in vivo* observations reflect intrinsic differences between osmolyte classes in their effects on protein stability *in vitro*. In the present study, we quantified the effects of eleven compounds from all three major chemical classes of protecting osmolytes and two denaturing osmolytes on the thermal stabilities of CRP and TNF α . For both proteins, there was no hierarchy of protective osmolyte effectiveness based on chemical class indicating that no one chemical class is intrinsically superior at enhancing thermal stability *in vitro* (compare molar m-values in Table 1 and Fig. S5). The lack of a strict chemical class hierarchy observed in this study is consistent with previous reports involving other protein targets including lysozyme and lactate dehydrogenase [4,44].

The rank order of osmolytes based on thermal stabilization effects was significantly different for the two protein systems, CRP and TNF α (compare molar m-values in Table 1 and Fig. S5). The most pronounced difference in the osmolyte responses of these proteins was to ectoine; this osmolyte was the most intrinsically effective stabilizer of CRP as



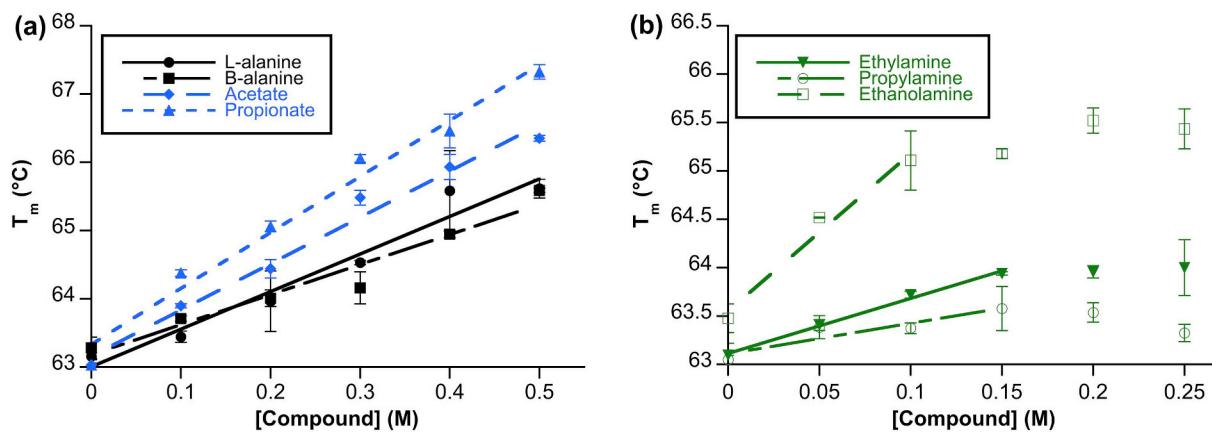


Fig. 7. CRP thermal stability measured over narrow concentration ranges to determine molar m-values. CRP T_m was measured at low concentrations of the amino acids L-alanine and β -alanine, and compounds lacking the amino group (acetate and propionate) (a) or lacking the carboxylate group (ethylamine, propylamine and ethanolamine) (b). The molar m-value for each osmolyte was determined by linear regression. For ethylamine, propylamine and ethanolamine, data points outside the linear range were excluded from the linear fit, as shown. Error bars represent the standard deviation of three replicates.

Table 3
Compound effects on CRP thermal stability.

Osmolyte	m-value ($^{\circ}\text{C}/\text{M}$) ^a
L-alanine	5.5
β -alanine	4.4
Acetate	6.8
Propionate	8.2
Ethylamine	5.7
Propylamine	3.1
Ethanolamine	16.3

^a Molar m-value, calculated from the slope of the plot of T_m versus molar test compound concentration, as shown in Fig. 7.

indicated by its molar m-value, but had no measurable effect on TNF α thermal stability at concentrations below 1 M and functioned to destabilize TNF α at higher concentrations (Fig. 2, Fig. 3, Table 1 and Fig. S4). Protein-specificity of the osmolyte effect has been reported previously. Di-myoinositol phosphate (DIP), for example, was shown to thermally destabilize lactate dehydrogenase. In contrast, DIP had no apparent effect on hydrogenase or ferredoxin oxidoreductase, but had a stabilizing effect on glyceraldehyde 3-phosphate dehydrogenase [4,45,46]. Results from the present study of the dissection of the functional group requirements for amino acid osmolytes also support the conclusion that solute effects on protein stability are highly protein-specific. For myoglobin, both the amino and carboxylate moieties of the amino acid osmolyte were required for protein thermal stabilization; removal of either functional group resulted in protein-destabilizing effects, and substitution of the carboxylate with a hydroxyl was not able to restore stabilizing activity (Fig. 5 and Table 2). Our results for myoglobin were consistent with those previously reported for AP-alcoholase, which also suggested that the complete amino acid zwitterion is required for thermal stabilization [16]. In distinct contrast with the results for myoglobin, compounds that lacked the amino group (acetate and propionate) functioned as thermal stabilizers of CRP and were, in fact, more effective than the complete, zwitterionic amino acids (L-alanine and β -alanine) in stabilizing this protein (Fig. 6, Fig. 7 and Table 3). Previous studies have shown that acetate (which occupies a central position in the Hofmeister series) modestly increases the thermal stability of lysozyme [47], but how widespread this phenomenon is amongst different protein model systems remains to be determined. Taken together, the results from Parts I and II of this study strongly support the conclusion that the relative effects of osmolytes or other small solutes on protein thermal stability are highly protein-

specific and indicate that the effects of some compounds may be stabilizing, or destabilizing, depending on the specific protein target.

For both CRP and TNF α , the mass-per-volume m-values differed dramatically for the osmolytes included in this investigation (Table 1, Fig. S6, Fig. S7 and Fig. S8). Consistent with this observation, there was no correlation between osmolyte molar m-values and osmolyte total accessible surface area for either protein model (data not shown). We also explored the possibility that differences in molar mass might explain apparent differences in stabilization activities of osmolytes within the same chemical class. For the amino acids, methylamines and denaturants, however, osmolytes within each chemical class revealed significantly different mass-per-volume m-values for both CRP and TNF α (Table 1, Fig. S6, Fig. S7 and Fig. S8). In contrast, all mono- and disaccharides in this study demonstrated similar mass-per-volume m-values with respect to TNF α stabilization, suggesting that apparent differences in carbohydrate-induced protein-stabilizing activity may be explained by differences in molar mass. This observation is consistent with previous reports that the stabilizing effects of carbohydrates increase with the number of covalently linked monosaccharide constituents [25,48]. However, our measurements of carbohydrate effects on CRP thermal stability were not consistent with this trend as revealed by dissimilar mass-per-volume m-values (Table 1 and Fig. S6a). Taken together, these differences in mass-per-volume m-values amongst osmolytes with respect to CRP and TNF α stabilization support the conclusion that consideration of molar mass, alone, is insufficient to explain differential osmolyte effects on protein thermal stability.

Previous measurements of groupwise transfer free energy values of amino acid side chains and polypeptide backbone units from water to osmolyte solutions have led to the prevailing view that interactions between osmolytes and the polypeptide backbone dominate the energetics that determine whether a particular osmolyte functions as a stabilizer or destabilizer [1,2,7,49]. Our results support previous studies that indicate that considerations of the solvent accessibility of the polypeptide backbone, alone, is insufficient to explain osmolyte effects e.g. [9,10]. If polypeptide backbone accessibility in the native versus denatured states was the only relevant protein component, the rank order of osmolyte effects would be predicted to be: TMAO > TMG > sucrose [9,10,26]. This rank order did not match the experimental rank order for either protein model: CRP (TMAO > sucrose > TMG); TNF α (sucrose > TMAO > TMG). This point is further emphasized by the observation that fractional change in backbone SASA in going from the native to denatured protein conformation is very similar for CRP and TNF α (compare Tables S7 and S8).

Our results indicated that the rank order of osmolytes predicted by m-value calculations using the Tanford transfer model [9,10] matched

the experimentally determined rank order for CRP, but not for TNF α . Caution must be taken when interpreting these results since the number of osmolytes for which these comparisons could be made in this study is small, due to the limited transfer free energy values available in the literature. For both protein models, the predicted rank order of protecting osmolyte effectiveness was TMAO > sucrose > TMG. The experimentally observed rank order for TNF α was sucrose > TMAO > TMG. The reasons for this difference are unclear. It is possible that the observed difference between the empirical and predicted rank ordering of osmolytes with respect to TNF α is due to the fact that the predicted rank order is based on transfer free energy values measured at 25 °C, whereas our empirical rankings are based on thermal melting temperatures. Because of the change in heat capacity associated with the unfolding transition of most proteins, and the fact that the free energy of unfolding is generally temperature-dependent, it is possible that the rank order of osmolytes reported in this study would be different if based on the free energy of unfolding at 25 °C. It is also possible that TNF α may have unique properties that account for its differential response to osmolytes. Studies have shown that osmolyte effects on protein folding and conformational stability are different for certain intrinsically disordered proteins compared to their effects on globular proteins [50,51]. Therefore, we analyzed CRP, TNF α and myoglobin for predicted regions of intrinsic disorder or flexibility to determine if differences exist between these proteins that could account for their differential responses to osmolytes or other small compounds [52] (Fig. S10). This analysis revealed that CRP and myoglobin have very little structural disorder or flexibility. (CRP has only a single C-terminal amino acid that is predicted to have significant structural flexibility while myoglobin has only one C-terminal amino acid with a significant disorder propensity and four C-terminal amino acids predicted to have significant structural flexibility). The soluble form of TNF α (as used in this study), however, has eight N-terminal amino acids with significant disorder propensity and eleven central amino acids predicted to have significant structural flexibility. It is unknown if these slight differences in structural disorder/flexibility amongst these proteins account for their differential responses to osmolytes and other small solutes; future studies are required to resolve this question.

In summary, the results from this study indicate that there is no intrinsic hierarchy amongst the protective osmolyte classes with respect to protein thermal stability. These findings are also consistent with previous observations that osmolyte effects are highly protein-specific. Furthermore, the results from this study also support the findings from previous studies that osmolyte effects cannot be fully explained by interactions with the protein backbone alone, but must also factor in other components of the protein fabric. These findings are particularly relevant to efforts to develop osmolyte-based chemistries for broad spectrum protein stabilization in patient biospecimens or complex reagent mixtures e.g. [53] and underscore the need to measure differential effects of osmolytes on the stabilities of the component proteins.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bpc.2020.106410>.

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