New light on protein folding: Unraveling folding and unfolding mechanisms using time-resolved and two-dimensional vibrational spectroscopy
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Despite its wide use as a denaturant, the mechanism by which guanidinium (Gdm\textsuperscript{+}) induces a protein to unfold remains a matter of controversy. In this chapter, we show evidence that Gdm\textsuperscript{+} can efficiently induce denaturation by disrupting salt bridges that stabilize the folded conformation. We study the Gdm\textsuperscript{+}-induced denaturation of a series of \(\alpha\)-helical peptides that contain pairs of positively (Arg or Lys) and negatively charged (Glu) residues, spaced and ordered in the sequence such that they form salt bridges that either stabilize, or destabilize the folded conformation. The peptides containing structure-stabilizing salt bridges are found to be denatured at much lower Gdm\textsuperscript{+} concentration than the peptides containing destabilizing salt bridges. In fact, for the latter, adding Gdm\textsuperscript{+} up to about 1 M concentration slightly increases the fraction of folded peptide. From the two-dimensional infrared spectrum of Gdm acetate in water, we find that Gdm\textsuperscript{+} binds directly to carboxylate groups. Hence, if a carboxylate group is involved in a salt bridge, competitive binding of the Gdm\textsuperscript{+} can disrupt this salt bridge. This explains both the observed efficient Gdm\textsuperscript{+} denaturation of the peptides containing stabilizing salt bridges, and the stabilizing effect of Gdm\textsuperscript{+} on the peptides containing destabilizing salt bridges. Binding of Gdm\textsuperscript{+} to Arg probably plays a minor role, since the same effects are observed for Glu/Arg and Glu/Lys salt bridges. The data can be modeled quantitatively by assuming specific binding of Gdm\textsuperscript{+} to the Glu carboxylate groups, and weaker, non-specific binding of Gdm\textsuperscript{+} to the remainder of the peptides. Our results indicate that one of the mechanisms underlying the protein-denaturing activity of Gdm\textsuperscript{+} involves its competitive binding to carboxylate side groups involved in salt bridges that stabilize the native conformation.
5.1 Introduction

Guanidinium chloride (GdmCl) is a widely used denaturing agent that is often used to investigate protein structure and activity. Despite extensive investigations into the denaturation activity of the guanidinium ion (Gdm$^+$) [1–19], at present a comprehensive molecular theory that can explain the underlying mechanism of its action has not yet been established. Different mechanisms have been proposed, involving direct effects in which Gdm$^+$ favorably interacts with specific protein groups [6, 7, 9, 11, 13, 17], indirect effects mediated by Gdm$^+$-induced perturbation of the hydrogen-bonding network of water [8, 16, 20], or a combination of both [18]. Recent experimental studies have provided ample evidence that Gdm$^+$ ions interact very weakly with water molecules [21, 22], suggesting that the direct mechanism is the primary factor in determining the denaturation activity of Gdm$^+$. As there exists a variety of ways in which Gdm$^+$ may bind to various chemical moieties in proteins [4], the question arises as to the nature and specificity of the Gdm$^+$/protein interactions that contribute to denaturation.

There is conflicting evidence as to whether Gdm$^+$ ions denature proteins by forming contacts with the amide groups of the polypeptide backbone or with specific amino-acid side chains. The ability of Gdm$^+$ to unfold α-helical model peptides that lack hydrophobic effects and significant structure-stabilizing contributions from specific side-chain groups [3, 4, 11, 13] suggests that Gdm$^+$ interacts with peptide groups, possibly by hydrogen-bonding to the peptide carbonyl groups, or by stacking interactions against the planar π-bonded peptide unit. In contrast, recent acid- and base-catalyzed hydrogen-exchange measurements on a small-molecule peptide model have shown that the Gdm$^+$ ion does not act on amide backbone groups [10]. In line with this finding, several other studies have indicated that preferential interaction of Gdm$^+$ with specific amino-acid side chains rather than with the backbone primarily accounts for its denaturant activity [6, 7, 12, 14, 23, 24]. Small-angle neutron scattering and molecular dynamics (MD) simulations suggest that Gdm$^+$ has the tendency to stack against aromatic [6, 11] and/or aliphatic [6] side chains, thereby disrupting favorable hydrophobic contributions to protein stability [19]. On the other hand, it has been observed in MD simulations that Gdm$^+$ perturbs electrostatic interactions much more effectively than hydrophobic contacts by interacting directly with negatively charged side groups [7]. The potential role of electrostatic interactions in Gdm$^+$-induced protein-denaturation was also demonstrated by circular dichroism (CD) experiments on two coiled-coil peptides that had a similar hydrophobic packing but different electrostatic interactions, and that were differently affected by Gdm$^+$ [2]. In addition, there is a strong indication from capillary electrophoresis [15] and MD simulations [6, 15, 23, 25, 26] that Gdm$^+$ in aqueous solution exhibits an affinity for the positively charged Gdm$^+$ moiety of arginine (Arg) side-chain groups, resulting in Gdm$^+$-Gdm$^+$ stacking interactions. The question thus arises if and how Gdm$^+$ affects electrostatic interactions between oppositely charged amino-acid side chains (salt bridges), which are known to play an essential role in the conformational stability of many proteins. To address this question, we here report
on a systematic investigation of the interaction of Gdm$^+$ with peptides containing oppositely charged amino-acid side chains involved in salt bridges. We study the effect of Gdm$^+$ on the helicity and conformational stability of alanine-based $\alpha$-helical peptides that have the same amino-acid composition, but differ in their salt bridge-forming capabilities due to differences in the sequence order. Our results indicate that Gdm$^+$ can efficiently denature the folded conformation by breaking up salt bridges through competitive binding with salt-bridge forming residues, in particular with carboxylate side groups involved in salt bridges.

5.2 Results and discussion

5.2.1 Gdm$^+$-induced unfolding of $\alpha$-helical model peptides

The sequences of the investigated alanine-based $\alpha$-helical peptides are listed in Table 5.1, together with their abbreviated names which indicate the distance (either 3 or 2 residues apart) and order of the salt-bridge forming residues. Depending on the spacing and order of the oppositely charged residues, they can form salt bridges that stabilize or destabilize the folded state (see section 4.2.2) [27, 28]. In particular, the salt bridges in the $(i + 4)$ER and $(i + 4)$EK peptides provide significant stabilization to the folded, helical conformation, whereas the salt bridges in the $(i + 3)$RE and $(i + 3)$KE peptides have the opposite effect and stabilize the unfolded conformation [27].

Fig. 5.1a shows CD spectra of peptide $(i + 4)$ER (in which the $\alpha$-helical structure is stabilized by the E/R salt bridges) at increasing concentrations of Gdm$^+$. In absence of Gdm$^+$, the CD spectrum exhibits the two minima characteristic for $\alpha$-helical structure at 208 and 222 nm (see section 2.1.1). Addition of increasing amounts of Gdm$^+$ causes a gradual decrease in the CD signal at 222 nm, reflecting the unfolding of the $\alpha$-helical structure (the minimum at 208 nm could not be reliably monitored in the presence of Gdm$^+$ which absorbs at wavelengths $< 210$ nm). As can be seen from the resulting denaturation profile in the inset of Fig. 5.1a, the presence of even relatively low concentrations of Gdm$^+$ already reduces the $\alpha$-helical population significantly, reflecting a strong sensitivity to the denaturant. Fig. 5.1c shows the temperature dependence of the helix content for three different Gdm$^+$ concentrations. These thermal unfolding curves have a sigmoidal shape, and from a singular-value decomposition (SVD) and global-fitting analysis we find

<table>
<thead>
<tr>
<th>Sequence$^a$</th>
<th>Name</th>
</tr>
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<tbody>
<tr>
<td>Ac-A(EAAAR)$_3$A-NH$_2$</td>
<td>$(i + 4)$ER</td>
</tr>
<tr>
<td>Ac-A(ARAAE)$_3$A-NH$_2$</td>
<td>$(i + 3)$RE</td>
</tr>
<tr>
<td>Ac-A(EAAAK)$_3$A-NH$_2$</td>
<td>$(i + 4)$EK</td>
</tr>
<tr>
<td>Ac-A(AKAAE)$_3$A-NH$_2$</td>
<td>$(i + 3)$KE</td>
</tr>
</tbody>
</table>
Guanidinium-induced denaturation by breaking of salt bridges

Figure 5.1: Low-temperature UV-CD spectral changes with increasing Gdm$^+$ concentration of (a) peptide $(i + 4)$ER (containing stabilizing salt bridges) and (b) peptide $(i + 3)$RE (containing destabilizing salt bridges). Due to the high absorption of Gdm$^+$ at short wavelengths, no CD data at short (<210 nm) wavelength can be obtained. The insets show the ellipticity at 222 nm versus Gdm$^+$ concentration. The solid curves represent a global least-squares fit to Eq. 5.1. Thermal unfolding curves of (c) peptide $(i + 4)$ER and (d) peptide $(i + 3)$RE detected at 222 nm with increasing levels of added Gdm$^+$. The solid curves are a least-squares fit to a two-state model (eq. 2.2) [30].

melting temperatures of $T_m = 312.9 \pm 1.2$ K (0 M Gdm$^+$), $T_m = 302.6 \pm 2.1$ K (0.4 M Gdm$^+$), and $T_m = 291.9 \pm 2.5$ K (1.5 M Gdm$^+$), showing that increasing levels of Gdm$^+$ result in a significant reduction in the thermal stability of the folded state. Interestingly, the melting temperature at the highest Gdm$^+$ concentration corresponds quantitatively to the melting temperature of 295.7±1.4 K observed for this peptide when its salt bridges are broken by completely protonating the Glu carboxylate groups [29]. These identical melting temperatures already hint at a denaturation mechanism in which Gdm$^+$ breaks the salt bridges.

Figures 5.1b and d show the UV-CD data of peptide $(i + 3)$RE, in which the $\alpha$-helical conformation is destabilized by the salt bridges (i.e., the salt bridges can form only if the helix is unfolded). As can be seen in Fig. 5.1b, the Gdm$^+$-induced CD changes of peptide $(i + 3)$RE indicate that the $\alpha$-helix remains intact with increasing Gdm$^+$ concentrations up to $\approx$1.5 M. Addition of modest concentrations of Gdm$^+$ even slightly increases the helical content (see the inset of Fig. 5.1b). We confirmed this small, but significant stabilization of the folded conformation upon adding Gdm$^+$ by measuring the melting temperatures. SVD and global fitting analysis of the thermal unfolding curves of peptide $(i + 3)$RE at different
levels of Gdm\(^+\) indeed reveal melting temperatures of \(T_m = 270.0 \pm 2.3\) K (0 M Gdm\(^+\)), \(T_m = 275.9 \pm 1.0\) K (0.4 M Gdm\(^+\)), and \(T_m = 276.3 \pm 1.0\) K (1.5 M Gdm\(^+\)). Further addition of Gdm\(^+\) (>1.5 M) eventually causes denaturation of the \(\alpha\)-helical structure. Comparison of the Gdm\(^+\) denaturation curves of peptides \((i + 3)\)RE and \((i + 4)\)ER (insets of Fig. 5.1b and 5.1a, respectively) reveals significantly different functional shapes, and shows that peptide \((i + 3)\)RE is considerably more resistant to Gdm\(^+\) denaturation than peptide \((i + 4)\)ER. A plausible explanation for this observation might be that Gdm\(^+\) binds to the salt-bridging side groups, thereby inhibiting the formation of structure-destabilizing, RE-oriented salt bridges and inducing a shift of the folding-unfolding equilibrium towards the folded, \(\alpha\)-helical state. A further increase of the Gdm\(^+\) concentration (>1.5 M) eventually results in denaturation of the folded state, probably due to binding of Gdm\(^+\) to the amide groups of the backbone. This latter effect counteracts the stabilizing effect caused by Gdm\(^+\) inhibiting the destabilizing salt bridge, and at sufficiently high Gdm\(^+\) concentration causes the peptide to unfold.

The difference in sensitivity to Gdm\(^+\) of the peptides with helix-stabilizing and destabilizing Glu\(^-\)/Arg\(^+\) salt bridges suggests that Gdm\(^+\) can cause denaturation by breaking salt bridges through interaction with one or both of the amino-acid side groups involved in the salt bridges. Gdm\(^+\) may interact with the negatively charged carboxylate (COO\(^-\)) side groups of Glu [2, 7, 14], but the salt bridges might also be disrupted due to stacking interactions between Gdm\(^+\) ions and the Arg side chain [6, 15, 23, 25, 26]. To investigate which specific side chain, Glu\(^-\) or Arg\(^+\), determines the denaturant sensitivity, we performed Gdm\(^+\)-induced unfolding measurements on similar helical peptides in which the Arg residues were replaced by Lys. MD simulations have demonstrated that Gdm\(^+\) ions do not bind to the positively charged side groups of Lys [15, 23].

Figure 5.2 shows the effect of adding Gdm\(^+\) to the peptides containing Glu\(^-\)/Lys\(^+\) salt bridges. We observe that the addition of increasing levels of Gdm\(^+\) considerably reduces the intensity of the CD signal at 222 nm of the salt-bridge optimized variant \((i + 4)\)EK, showing that the \(\alpha\)-helix is denatured by Gdm\(^+\) (Fig. 5.2a), just like the Arg-based peptide. The Gdm\(^+\) denaturation curve of peptide \((i + 4)\)EK is very similar to that of \((i + 4)\)ER (see the insets of Fig. 5.2a and 5.1a, respectively). Furthermore, we find melting temperatures of \(T_m = 292.6 \pm 0.7\) K (0 M Gdm\(^+\)), \(T_m = 284.3 \pm 1.4\) K (0.4 M Gdm\(^+\)), and \(T_m = 283.8 \pm 2.6\) K (1.5 M Gdm\(^+\)), showing that addition of Gdm\(^+\) significantly reduces the conformational stability of the \((i + 4)\)EK peptide (Fig. 5.2c). In the peptide \((i + 3)\)KE containing conformation-destabilizing Glu\(^-\)/Lys\(^+\) salt bridges, we observe that the peptide maintains its folded conformation up to Gdm\(^+\) concentrations of 0.75 M (Fig. 5.2b), indicating a considerable lack of denaturant activity of Gdm\(^+\) against peptide \((i + 3)\)KE. Again, the functional shape of the Gdm\(^+\) denaturation curve of the Lys-based peptide is similar to that of the Arg-containing variant (see the insets of Fig. 5.2b and 5.1b, respectively). The melting temperatures of peptide \((i + 3)\)KE with increasing Gdm\(^+\) concentration are \(T_m = 274.8 \pm 1.5\) K (0 M Gdm\(^+\)), \(T_m = 280.3 \pm 2.0\) K (0.4 M Gdm\(^+\)), and \(T_m = 277.6 \pm 4.7\) K (0.75 M Gdm\(^+\)). Thus, also in this case, the addition of Gdm\(^+\) (in modest concentrations)
enhances the fold stability of peptide \((i+3)\)KE: the presence of Gdm\(^+\) ions causes disruption of the unfavorable KE-oriented salt bridges by interacting directly with the salt-bridging side groups. The observation of similar effects of Gdm\(^+\) on the conformational stability of the Arg- and Lys-based peptides indicates that Gdm\(^+\) interacts predominantly with the COO\(^-\) side groups of Glu. Our data suggest that the Gdm\(^+\) sensitivity is slightly greater in Lys-based variants, as denaturation of peptide \((i+3)\)KE sets in at a slightly lower Gdm\(^+\) concentration than that of peptide \((i+3)\)RE. This difference probably arises from differences in the salt-bridge formation probability and/or differences in the strength of salt-bridge interactions formed between Glu\(^-\)/Lys\(^+\) and Glu\(^-\)/Arg\(^+\).

### 5.2.2 Quantitative analysis

We can quantitatively describe the denaturation data (shown in the insets of Figs. 5.1a,b and 5.2a,b) using a model that takes into account both the specific binding of Gdm\(^+\) to the Glu carboxylate (disrupting the salt bridges), and its weaker, non-specific binding to the remainder of the peptide. The quantitative description of this type of model is well established [31]. The effect of Gdm\(^+\) on the folding equilibrium arises from the fact that it has different association constants.
for binding to the peptide in its folded and unfolded states. The dependence of the free-energy change $\Delta G_{\text{unf}}$ of the folded→unfolded transition on the Gdm$^+$ concentration $C$ is then given by [31]:

$$
\Delta G_{\text{unf}}(C) = \Delta G_{\text{unf}}^0 - n_{\text{sites}}RT \ln(1 + K_u C) - \ln(1 + K_f C) - m C,
$$

(5.1)

where $\Delta G_{\text{unf}}^0$ is the free energy of unfolding in absence of Gdm$^+$, $C = [\text{Gdm}^+]$, $n_{\text{sites}} = 3$ the number of binding sites (in this case Glu carboxylate groups [32]) in the peptide, $R$ the gas constant, $T$ the temperature, $K_u$ and $K_f$ the association constants for Gdm$^+$:Glu$^-$ binding in the unfolded and folded states respectively, and $m$ a constant. The second term accounts for the specific binding of Gdm$^+$ to the Glu carboxylate groups; the third term for the non-specific binding of Gdm$^+$, with $m$ being typically on the order of 1 to a few kcal mol$^{-1}$ M$^{-1}$ for globular proteins [33, 34]. The $(i + 4)\text{ER}$ and $(i + 4)\text{EK}$ peptides have salt bridges in the folded state, but not in the unfolded state. Hence, for these peptides $K_u > K_f$ (since in the folded state the Glu$^-$/Arg$^+$ or Glu$^-$/Lys$^+$ salt bridges compete efficiently with Glu:Gdm$^+$ binding whereas in the unfolded state this does not happen). Hence for these peptides, the second and third terms in Eq. 5.1 are both negative so that $\Delta G_{\text{unf}}$ decreases (and the peptide starts to unfold) even at the lowest Gdm$^+$ concentrations. In the $(i + 3)\text{RE}$ and $(i + 3)\text{KE}$ peptides on the other hand, salt bridges are present in the unfolded state but cannot be formed in the folded state, so that $K_f > K_u$ (since in the unfolded state, Glu$^-$:Gdm$^+$ binding has to compete with Glu$^-$/Arg$^+$ or Glu$^-$/Lys$^+$ salt-bridge formation), and the second term in Eq. 5.1 is positive, leading to an increase of $\Delta G_{\text{unf}}$ with increasing Gdm$^+$ concentration. However, for sufficiently high Gdm$^+$ concentration this increase is counter-acted by the weaker, non-specific binding of Gdm$^+$ to the peptide, represented by the third term, which dominates at high Gdm$^+$ concentrations. This explains our observation that in the $(i + 3)\text{RE}$ and $(i + 3)\text{KE}$ peptides, the helical conformation is stabilized at low Gdm$^+$ concentration, but is denatured at higher Gdm$^+$ concentration. We find that we can quantitatively describe our data by assuming that the Glu:Gdm$^+$ association constant in the absence of competing salt bridges (i.e., in the unfolded state of peptides $(i + 4)\text{ER}$ and $(i + 4)\text{EK}$, and in the folded state of peptides $(i + 3)\text{RE}$ and $(i + 3)\text{K}$) is equal to the association constant 0.37 M$^{-1}$ of Gdm$^+$ and acetate in water [35]. The Gdm$^+$-carboxylate association constants in the presence of competing salt bridges are unknown, as are the value of $m$. These values are obtained from simultaneous least-squares fits to the $(i + 4)\text{ER}$ and $(i + 3)\text{RE}$, and to the $(i + 4)\text{EK}$ and $(i + 3)\text{KE}$ peptides, respectively. The sets of Arg- and Lys-based peptides are treated independently, as it may be expected that the Gdm$^+$ association constant in the presence of competing salt bridges depends on whether these competing salt bridges are formed with Arg or with Lys. From the least-squares fits (the results of which are shown as the curves in the insets of Figs. 5.1a and b), we obtain for the association constants in presence of salt bridges 0.03 ± 0.02 M$^{-1}$ for the Arg-based peptides, and 0.09 ± 0.03 M$^{-1}$ for the Lys-based peptides. As expected, both these values are much lower than the association constant in the absence of salt bridges (0.37 M$^{-1}$) [35]. The lower association constant in the presence of
Glu−/Arg+ salt bridges as compared to Glu−/Lys+ salt bridges indicates that the Glu−/Arg+ salt bridges are stronger (and so less easily broken up by Gdm+) than the Glu−/Lys+ salt bridges, in agreement with the lower melting temperature of the peptides containing the latter. For the parameter $m$ characterizing the non-specific binding we obtain $500 \pm 40$ and $530 \pm 40$ cal mol$^{-1}$ M$^{-1}$ for the Arg and Lys-based peptides, the similarity of the values confirming the non-specific character of the Gdm+ binding described by the third term in Eq. 5.1.

### 5.2.3 2D-IR investigation of aqueous Gdm+/COO− interactions

To date, the association of Gdm+ with carboxylate groups in aqueous solution has only been studied indirectly, in particular through the effect of association on the carboxylate p$K_a$ [35, 36]. These studies concluded that Gdm+/COO− association occurs with an association constant on the order of 0.5 M$^{-1}$. To examine in a more direct manner if Gdm+ binds to COO− groups, we have performed 2D-IR spectroscopy on a deuterated aqueous solution of guanidine acetate (GdmDAc). We recently demonstrated that 2D-IR spectroscopy provides direct experimental evidence for the formation of salt-bridged dimers of Gdm+ and Ac− in dimethylsulfoxide (DMSO), by detecting the vibrational coupling between the two CN$_3$D$_6^+$ modes of Gdm+ to the COO−-stretch mode of Ac− [37], and similar measurements have been used to study N-methylacetamide dimers [38]. Here, we investigate if the association of Gdm+ and COO− in aqueous solution can be observed using 2D-IR spectroscopy. Fig. 5.3a presents the infrared absorption spectra of isolated Gdm+ (GdmDCl, cyan) and Ac− (NaAc, purple). The spectrum of Gdm+ shows a broad absorption peak centered at 1600 cm$^{-1}$, arising from a degenerate CN$_3$D$_6^+$ mode due to a combined CN$_3$ antisymmetric stretch and ND$_2$ scissors motion [39]. The frequency of the COO−-antisymmetric-stretch mode of Ac− is 1560 cm$^{-1}$. Upon mixing equimolar NaAc and GdmDCl solutions, we observe frequency shifts of the infrared bands of the Gdm+ and Ac− ions (see Fig. 5.3d, where we compare the spectrum of GdmD.Ac to that constructed by adding the spectra of equimolar GdmDCl and NaAc solutions): the CN$_3$D$_6^+$ frequency of Gdm+ increases by 2 cm$^{-1}$, while the COO−-antisymmetric-stretch frequency decreases by 2 cm$^{-1}$. This increase in the frequency splitting between two vibrational modes suggests that the modes are coupled [40]. To confirm this, 2D-IR measurements were performed on deuterated aqueous solutions of GdmDCl, NaAc, and GdmD.Ac, see Figure 5.3. To compare the 2D-IR response of isolated Gdm+ and Ac− ions and that of a solution containing Gdm+/Ac− dimers, 2D-IR spectra of a (hypothetical) mixture of monomeric Gdm+ and Ac− were constructed by adding the individual experimental 2D-IR responses of NaAc and GdmDCl solution (see Fig. 5.3b and c). We observe a cross-peak pair at $(\nu_{\text{probe}}, \nu_{\text{pump}}) = (1590, 1610)$ and (1612, 1588) cm$^{-1}$ in the 2D-IR difference spectrum that is constructed of isolated Gdm+ and Ac− ions. This cross-peak pair originates from the coupling between the two CN$_3$D$_6^+$ modes of Gdm+ (Fig. 5.3c) [37]. By contrast, the 2D-IR polarization-difference spectrum of the GdmD.Ac solution displays two additional cross-peak features between the CN$_3$D$_6^+$ and COO− modes, see Fig. 5.3e.
Figure 5.3: (a) Normalized infrared absorption spectra of solutions of isolated Gdm$^+$ (GdmDCl) and Ac$^-$ (NaAc), both at 1.5 M concentration in D$_2$O (solvent subtracted). The dashed spectrum is the sum of the Gdm$^+$ (GdmDCl) and Ac$^-$ (NaAc) spectra. (b) Constructed 2D-IR spectrum obtained by adding the 2D-IR spectra of isolated Gdm$^+$ and Ac$^-$ ions. This is the spectrum expected if no interaction between Gdm$^+$ and Ac$^-$ would occur. Blue indicates negative absorption change, red positive absorption change. (c) Polarization difference 2D-IR spectrum ($2.8 \Delta A_{\perp} - \Delta A_{\parallel}$), constructed in the same manner as (b). (d) Normalized IR spectrum of 1.5 M GdmAc solution in D$_2$O (solvent subtracted). (e) 2D-IR spectrum of this solution. (f) Polarization difference 2D-IR spectrum ($2.8 \Delta A_{\perp} - \Delta A_{\parallel}$) of this solution. All 2D-IR spectra were obtained a pump-probe delay of 1 ps.
The cross-peak features overlap with the much intenser diagonal peaks, and can be seen better in the polarization-difference spectrum [37], shown in Fig. 5.3f. The Gdm$^+/\text{COO}^-$ cross-peak response, which is very similar to that observed for Gdm$^+:\text{Ac}^-$ dimers in DMSO [37], indicates that the CN$_3$D$_6^+$ modes of Gdm$^+$ are vibrationally coupled to the COO$^-$-stretch mode of Ac$^-$ due to association of the two ions. We observe these aqueous Gdm$^+/\text{COO}^-$ interactions only at relatively high concentrations (>0.75 M), which is in agreement with the reported low equilibrium constants for association ($K_a$) of Gdm$^+$ and Ac$^-$ in aqueous solution [35, 36].

5.2.4 Gdm$^+$-induced unfolding of the Trp-cage miniprotein

To gain insights into the molecular basis of Gdm$^+$-induced protein-denaturation processes in the simultaneous presence of stabilizing salt bridges and hydrophobic tertiary interactions, the UV-CD measurements were extended to the 20-residue Trp-cage miniprotein. Trp-cage's folded conformation contains well-defined secondary and tertiary structure elements, including an N-terminal $\alpha$-helix, a short $3_{10}$-helix, a C-terminal polyproline II helix, and a compact hydrophobic cage structure that is formed by the packing of the aromatic side chains of Tyr3 and Trp6 against residues Gly11, Pro12, Pro18, and Pro19 (see Fig. 6.1) [41]. Enhanced stabilization of Trp-cage’s three-dimensional fold is provided through the formation of a salt bridge between residues Glu9 and Arg16. To identify potential interactions of Gdm$^+$ with charged and/or hydrophobic amino-acid side chains and to verify their relative importance, we study the protein-denaturing ability of Gdm$^+$ against a salt bridge-deficient (TC6a: NLYIQ WLK$^E$G GPSSG RPPPS) and a salt bridge-optimized (TC6b: NLYIQ W$^E$L K$^E$G GPSSG RPPPS) mutant, differing only by a single point mutation at position 9 (Asn, N $\rightarrow$ Glu, E) [42]. It has been shown previously that the mutation from Asn9 to Glu9 enables the formation of a salt bridge with residue Arg16, thereby providing enhanced fold stabilization to the native structure of TC6b [42].

Fig. 5.4 displays the Gdm$^+$-induced unfolding data of the salt bridge-deficient (TC6a) and salt bridge-optimized (TC6b) mutants of Trp-cage. Comparison of the UV-CD spectra of TC6a and TC6b collected in the absence of Gdm$^+$ (see Fig. 5.4a and b, respectively) show that the magnitude of the CD signal of the salt bridge-optimized mutant is significantly larger compared to that of the salt bridge-deficient mutant, reflecting different levels of structure stability due to the absence or presence of the Glu9–Arg16 salt bridge. As can be seen from the insets in Fig. 5.4a and b, for both mutants the ellipticity at 222 nm drops significantly upon the addition of increasing levels of Gdm$^+$, indicating structural denaturation.

The thermal denaturation curves in the presence of Gdm$^+$ at varying concentrations are shown in Fig. 5.4c (TC6a) and Fig. 5.4d (TC6b). In the absence of Gdm$^+$, we find transition midpoints of $T_m = 314.3 \pm 0.7$ K (TC6a) and $T_m = 322.7 \pm 1.0$ K (TC6b), reflecting the stabilizing effect provided by the salt-bridge interaction between Glu9 and Arg16. These values are significantly higher compared to the transition midpoints of 301.8 K (TC6a) and 310.7 K (TC6b) re-
Figure 5.4: Low-temperature UV-CD spectral changes with increasing Gdm$^+$ concentration of the Trp-cage mutants (a) TC6a (salt bridge-deficient) and (b) TC6b (salt bridge-optimized). The insets show the ellipticity at 222 nm versus Gdm$^+$ concentration. The solid curves represent a global least-squares fit to Eq. 5.1. Thermal unfolding curves of peptide (c) TC6a and (d) TC6b detected at 222 nm with increasing levels of added Gdm$^+$. The solid curves are a least-squares fit to a two-state model (eq. 2.2) [30].

ported previously in literature, however, the difference between the melting temperatures of TC6a and TC6b is similar [42]. The addition of increasing Gdm$^+$ levels causes a significant reduction in the thermal stability of both mutants: $T_m = 307.7 \pm 1.2$ K (0.4 M Gdm$^+$) and $T_m = 304.7 \pm 1.2$ K (1.5 M Gdm$^+$) for the salt bridge-deficient mutant (TC6a), and $T_m = 318.5 \pm 1.7$ K (0.4 M Gdm$^+$) and $T_m = 307.3 \pm 2.3$ K (1.5 M Gdm$^+$) for the salt bridge-optimized mutant (TC6b). Surprisingly, the melting temperature of TC6a decreases relatively faster upon increasing Gdm$^+$ concentration compared to TC6b, indicating that the salt bridge-deficient mutant shows a higher sensitivity to Gdm$^+$ denaturation than the salt bridge-optimized variant. At first sight, this might suggest that in the salt-bridge optimized mutant of the Trp-cage miniprotein (in contrast to the salt-bridge forming $\alpha$-helices) direct interactions between Gdm$^+$ ions and COO$^-$ side groups involved in salt bridges is not the primary mechanism underlying the denaturing activity of Gdm$^+$. However, another probable explanation for the unexpected high sensitivity of TC6a to Gdm$^+$ is that the single point mutation at position 9 has multiple structure-modifying effects. It has been shown previously that the Glu9–Arg16 salt bridge occupies an essential role in holding the three-dimensional fold of Trp-cage together, and that the elimination of this
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salt bridge has multiple structural consequences [42]. Accordingly, the mutation of Glu to Asn not only hinders the formation of a salt bridge, but it also alters the relative orientation of the aromatic side group of residue Tyr3 by turning it somewhat outward from the hydrophobic pocket [42]. As a result, the hydrophobic contacts formed between the aromatic rings of residues Tyr3 and Trp6 and the polyproline helix are rearranged, leading to a reduced compactness of the hydrophobic core structure in the absence of salt-bridge interactions [42]. Possibly, the accessibility of the hydrophobic pocket for Gdm$^+$ ions is therefore greater for the salt bridge-deficient mutant of Trp-cage than for the salt bridge-optimized variant. MD evidence indicates that besides the favorable interactions of Gdm$^+$ ions with the negatively charged COO$^-$ side group of residue 9 forming the salt bridge, stacking-interactions with the aromatic rings of Tyr3 and Trp6 play an important role in the Gdm$^+$-induced denaturation process of Trp-cage [14]. However, because these two structural properties are correlated for Trp-cage, this miniprotein is not a suitable model system for studying the role of salt-bridge and/or hydrophobic interactions in Gdm$^+$-induced denaturation.

5.3 Conclusions

Our experiments show a strong correlation between the presence of structure-stabilizing salt bridges in a peptide and its sensitivity to Gdm$^+$ denaturation, providing evidence that Gdm$^+$ can efficiently denature proteins by breaking salt bridges through competitive binding with COO$^-$ side groups. We find that Gdm$^+$-induced denaturation of peptides with structure-stabilizing Glu$^-$/Arg$^+$ and Glu$^-$/Lys$^+$ salt bridges is much more efficient compared to peptides that are not stabilized by salt bridges. Interestingly, the perturbation by Gdm$^+$ of destabilizing salt bridges even slightly increases the helicity of the peptides, suggesting that disruption of these structure-destabilizing salt bridges by Gdm$^+$ in these peptides shifts the folding equilibrium towards the folded state. Further addition of Gdm$^+$ eventually leads to the denaturation of the folded conformation of all investigated peptides, which suggests that besides breaking the salt bridges, Gdm$^+$ also interacts with backbone peptide groups, and that this interaction becomes dominant at high Gdm$^+$ concentrations. The observed similar Gdm$^+$-sensitivity of Arg- and Lys-containing salt bridges demonstrates that Gdm$^+$ binds to the COO$^-$ side groups of Glu rather than to the Arg side groups. The 2D-IR spectra of Gdm acetate show direct evidence for such aqueous Gdm$^+$:COO$^-$ association.

Our data are consistent with previous MD results [7], and provide direct experimental support to the notion that favorable electrostatic contributions to protein stability play a key role in Gdm$^+$-induced denaturation. In particular, our observations indicate that Gdm$^+$-induced unfolding of proteins and peptides can proceed efficiently through a direct mechanism in which Gdm$^+$ binds to COO$^-$ side groups involved in salt bridges, besides the weaker and less specific binding to the other side groups and/or the protein backbone.
5.4 Experimental details

The sequences of the investigated alanine-based α-helical peptides were Ac-A(AAAA\textsubscript{B})\textsubscript{3}A-NH\textsubscript{2} and Ac-A(ABAAA)\textsubscript{3}A-NH\textsubscript{2}, with Ac = acetyl, A = alanine, A = acidic residue (glutamic acid), B = basic residue (either arginine or lysine), see Table 5.1. Two different mutants of the Trp-cage miniprotein were studied, consisting of the sequences NLYIQ WL\textsubscript{K}NG GPSSG RPPPS (TC6a) and NLYIQ WL\textsubscript{K}\textsubscript{E}G GPSSG RPPPS (TC6b) \cite{42}. All peptides were custom-synthesized from GL Biochem (Shanghai). Peptide purity (≥95%) was assessed by reversed-phase HPLC, and the peptides were lyophilized against a 35% HCl/H\textsubscript{2}O solution to remove residual trifluoroacetic acid (TFA). Peptide solutions of 50 µM were prepared using a 20 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} buffer in H\textsubscript{2}O (pH = 7.0). Guanidine-HCl (≥ 99% purity) was purchased from Sigma-Aldrich, and aliquots of a 5.0 M stock solution were added to the peptide solutions to prepare a series of samples containing different Gdm\textsuperscript{+} concentrations (0 – 3.7 M). After the addition of guanidine-HCl, the pH of the peptide-solutions was adjusted to neutral pH level (pH = 7.0) by addition of NaOH solution (the volume added was less than 1% of the peptide solution).

UV-CD spectra and thermal unfolding curves were collected on a Chirascan-plus CD Spectrometer (Applied Photophysics) using 2-mm quartz cuvettes over a UV range of 190 – 265 nm with incremental steps of 1.0 nm and averaging over 2 s of data acquisition time. Each spectrum was obtained by averaging a total of at least five scans. The ellipticity at 222 nm was collected as indicator of α-helical structure, and CD band intensities were expressed in mean residue ellipticity. Temperature control was achieved using a Quantum Northwest (TC125) temperature controller in the range of 268 – 363 K. Before each measurement, the peptide solution was allowed to equilibrate for 5 min at the adjusted temperature prior to data acquisition. A direct temperature probe was inserted into the peptide solution for continuously monitoring of the actual sample temperature. Global fitting of the datasets collected as function of temperature were performed according to a two-state model (\textit{F} = \textit{U}).

The guanidinium-Ac (≥ 99% purity), guanidine-HCl (≥ 99% purity), and sodium acetate (≥ 99% purity) used in the 2D-IR experiments were all purchased from Sigma-Aldrich. H/D-exchange of the carboxyl and guanidinium groups was achieved by evaporating the compounds from excess D\textsubscript{2}O, and sample solutions of 1.5 M were prepared by directly dissolving the lyophilized solids in D\textsubscript{2}O. Fourier-transform infrared (FTIR) spectra were collected using a Bruker Vertex 70 spectrometer (2 cm\textsuperscript{-1} resolution). The samples were kept at 1.5 M concentration between 2 mm thick CaF\textsubscript{2} windows separated by a 8 µm Teflon spacer. A detailed description of the femtosecond pump-probe setup as used for the 2D-IR experiments has been reported previously \cite{43} (see section 2.1.2).
References

Based on the similarity of the Glu/Arg and Glu/Lys results, we assume that the association of Gdm$^+$ with Arg is much weaker than with the Glu carboxylate groups. Binding of Gdm$^+$ to Arg would not change the equation, but would cause an increase in the effective association constant.


