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Protein Denaturation with Guanidinium: A 2D-IR Study

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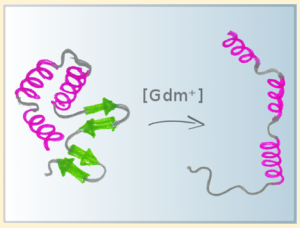
Supporting Information

ABSTRACT: Guanidinium (Gdm^+) is a widely used denaturant, but it is still largely unknown how it operates at the molecular level. In particular, the effect of guanidinium on the different types of secondary structure motifs of proteins is at present not clear. Here, we use two-dimensional infrared spectroscopy (2D-IR) to investigate changes in the secondary structure of two proteins with mainly α-helical or β-sheet content upon addition of Gdm-$^{13}C^{15}N_3$·Cl. We find that upon denaturation, the β-sheet protein shows a complete loss of β-sheet structure, whereas the α-helical protein maintains most of its secondary structure. These results suggest that Gdm^+ disrupts β-sheets much more efficiently than α-helices, possibly because in the former, hydrophobic interactions are more important and the number of dangling hydrogen bonds is larger.



SECTION: Biophysical Chemistry and Biomolecules

Significant effort has been dedicated to elucidating the molecular mechanism of the effect of chemical denaturants (reagents that decrease protein stability when added to the aqueous solvent), the most effective one of which is the guanidinium cation (Gdm^+).[1−3] There are two mechanisms by which a denaturant could destabilize proteins, indirectly by altering the solvent properties of water and directly by specifically interacting with groups of the protein. The indirect mechanism is most likely not applicable for Gdm^+ because there is ample experimental evidence that the interaction between water molecules and Gdm^+ is very weak.[4,5] However, Gdm^+ does seem to affect the water−water interactions in the hydration shell of other solutes,[6] and it has been proposed that the lack of a hydration shell of Gdm^+ promotes solubility of nonpolar groups in the interior of proteins.[4] Hence, Gdm^+ most likely denatures proteins by interacting directly with them, but it has been shown that Gdm^+ does not form hydrogen bonds with peptide groups.[7] The denaturation mechanism of Gdm^+ therefore involves interactions with the side chains of proteins, but the physical nature of these interactions is only just starting to become clear.[7−11]

At present, it is not known whether Gdm^+ disrupts certain types of protein secondary structure more efficiently than others as most studies to date focus on interactions between Gdm^+ and the side chains of proteins.[7−11] Addressing this question requires techniques that are sensitive to the secondary structure of proteins. Circular dichroism (CD), a commonly used probe of structure loss upon denaturation, is not so well suited for this purpose because it has a high sensitivity to α-helical content but a low one to β-sheet secondary structure. Moreover, a molecular interpretation of the CD data is often hindered by the responses of aromatic side chains to conformational variations. Infrared absorption spectroscopy (FTIR) provides a more direct probe of secondary structure. This is because the amide I′ band (mainly due to C═O vibrations of the backbone of the protein) is centered at distinctive frequencies depending on the secondary structure; an α-helix typically has two overlapping absorption bands centered at around 1650 cm^{-1}, a β-sheet has two bands centered at 1630 and 1670 cm^{-1}, and a random coil shows a broad band centered at around 1650 cm^{-1}. Despite the frequency specificity of the secondary structure motifs of proteins, conformational disorder and solvation effects often result in broad, featureless amide I′ bands. When a protein goes through a structural change, such as denaturation, the amide I′ response undergoes concomitant changes, but it is often difficult to disentangle the specific secondary structure contributions from the FTIR spectrum.

Here, we study the Gdm^+ denaturation of two proteins by directly probing their secondary structure using two-dimensional infrared spectroscopy (2D-IR). The 2D-IR response of the amide I′ band of proteins is very sensitive to the different secondary structure motifs.[12,13] By using 2D-IR spectroscopy, we selectively excite vibrational modes at a specific frequency in the amide I′ region with a narrow-band pump pulse and subsequently observe the response of the protein with a broad-band probe pulse. By scanning the pump frequency across the amide I′ band, we construct a two-dimensional graph. The diagonal response in this graph is analogous to the FTIR spectrum but has increased sensitivity and resolution.[12] Furthermore, off-diagonal response (cross peaks) arises when a coupling exists between two vibrational modes, such as between the two IR-active β-sheet modes. By changing the relative polarization of the pump and probe pulses, the visibility of the cross peaks can be enhanced. The increased sensitivity and resolution of the diagonal amide I′ response and the cross-peak patterns make 2D-IR spectroscopy more sensitive to secondary structure than the corresponding FTIR spectrum.[12]

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Studying Gdm^+ denaturation of proteins with IR is difficult because Gdm^+ absorbs at 1600 cm^{-1} due to a degenerate mode involving a combined CN_3 antisymmetric stretch and NH_2 scissor motion.[14] This band overlaps with the amide I′ band and overwhelms it at the Gdm^+ concentrations needed to denature proteins. Although it is in principle possible to subtract this contribution,[15] the conditions under which this subtraction is possible are limited. To avoid this problem, we use Gdm-$^{13}C^{15}N_3$·HCl (99 atom % ^{13}C, 98 atom % ^{15}N, Sigma-Aldrich). The absorption frequency of this isotope is red-shifted by about 60 cm^{-1} (see Figure S1, Supporting Information), leaving a clear spectral window around the amide I′ frequency.

We investigate two well-known proteins, lysozyme (from chicken egg white, Sigma-Aldrich), which is mainly α-helical, and α-chymotrypsin (from bovine pancreas, Sigma-Aldrich), which is mostly formed by β-sheets; see Figure 1. Both proteins

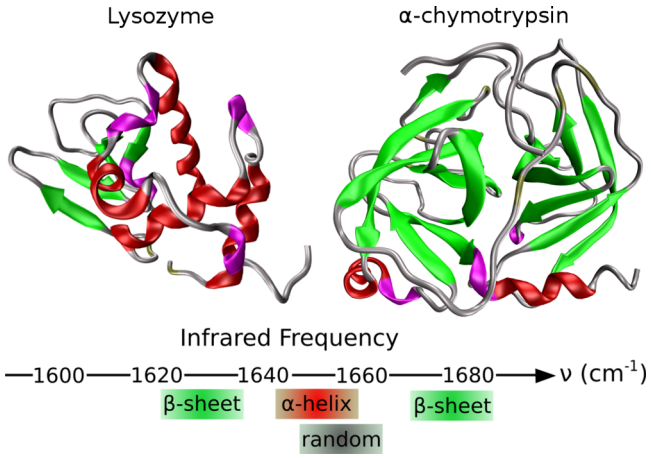

Figure 1. Structure of the investigated proteins. Lysozyme (PDB ID 2LYZ, 129 residues) shows 41% helical and 10% β-sheet secondary structure. α-Chymotrypsin (PDB ID 1YPH, 241 residues) shows 11% helical and 34% β-sheet secondary structure. β-sheets are shown in green, α helices in red, 3_{10} helices in pink, and random coils in gray. The infrared absorption frequencies of these secondary structure motifs are indicated below the proteins.

undergo an unfolding reaction upon addition of Gdm·Cl.[16,17] The native-state 2D-IR spectra of these proteins have been determined before,[13,18] and our spectra are in agreement with these earlier measurements. Here, we focus on the changes in the 2D-IR spectra of these proteins upon denaturation by Gdm^+ by measuring a series of 2D-IR spectra for increasing concentration of Gdm^+ around the transition point of the unfolding reaction. Experimental details can be found in the Supporting Information.

The FTIR spectra of native and denatured lysozyme (5 M Gdm^+ concentration) are shown in Figure 2A and D, respectively. The amide I′ band of denatured lysozyme blue shifts with the respect to the spectra of the native protein, indicating increased random coil content (this shift is more easily seen in Figure S2 (Supporting Information), where FTIR spectra for intermediate Gdm^+ concentrations are shown). Figure 2B shows the 2D-IR spectrum of folded lysozyme for perpendicular polarization of pump and probe pulses, and in Figure 2C, we show the 2D-IR polarization difference spectrum ($3\Delta\alpha_\perp - \Delta\alpha_\parallel$, which eliminates the diagonal peaks and leaves only cross peaks),[12] in which the coupled A and E_1 modes of the α-helix are visible. The frequency splitting between these modes is consistent with theoretical predictions[19] and previous 2D-IR measurements on α-helices.[20] Figure 2E shows the 2D-IR spectrum of Gdm^+-denatured lysozyme (see Figure S3 (Supporting Information) for intermediate concentrations of Gdm^+), where the blue shift of the amide I′ band is clear, confirming the larger amplitude at the random coil frequency. Surprisingly, the polarization difference spectrum, shown in Figure 2F, remains essentially the same as that of folded lysozyme. Figure 3A shows cross sections along the dashed line in these difference spectra for increasing concentration of Gdm^+, where little change is observed in the amplitude of the cross peak. In the inset of Figure 3A, we plot the amplitude of this cross peak (maximum of the positive part minus the minimum of the negative one, normalized to the value of folded lysozyme) as a function of Gdm^+ concentration, and it can be seen that it remains constant. This result demonstrates that α-helical structure remains upon denaturation of the protein by Gdm^+. Lysozyme has four disulfide bonds that stabilize the globular structure of the protein,[21] and Gdm^+ will not disrupt these tertiary contacts. Therefore, the increase in the random coil conformation that we observe is probably due to loss of the 10% β-sheet structure and of the turns as the protein unfolds and looses part of its tertiary structure. This interpretation agrees with optical rotation studies that showed that lysozyme still has a certain degree of intramolecular order and thus is not a completely random coil at a 5 M concentration of Gdm^+, although the denaturation reaction is completed.[16] Furthermore, it was shown recently that at this same Gdm^+ concentration, lysozyme maintains about 70% of its enzymatic activity.[22] This indicates that the remaining α-helical structure of lysozyme, partly kept together by the disulfide bonds, is probably sufficient for the significant activity of the protein.

α-Chymotrypsin shows a different behavior upon addition of Gdm^+. This protein completes a denaturation reaction at a 4 M Gdm^+ concentration.[16] The FTIR spectra of native and denatured α-chymotrypsin (4 M Gdm^+ concentration) are shown in Figure 2G and J, respectively. As for lysozyme, the amide I′ band of native and denatured α-chymotrypsin undergoes a blue shift, indicating increased random coil conformation (see Figure S2 (Supporting Information) for intermediate Gdm^+ concentrations). The perpendicular polarization 2D-IR spectrum of the native state of the protein, shown in Figure 2H, is dominated by the two coupled amide I′ modes of the β-sheet structure at 1620 and 1675 cm^{-1}.[18] These coupled modes give rise to a 2D-IR line shape that is typical of β-sheets due to the presence of strong cross peaks. The polarization difference 2D-IR spectrum, shown in Figure 2I, shows the isolated cross peaks between the β-sheet modes as well as a small α-helical contribution. At a Gdm^+ concentration of 4 M, the 2D-IR spectrum (shown in Figure 2K and L; see Figure S4 (Supporting Information) for intermediate concentrations) has lost this typical β-sheet line shape. The perpendicular polarization spectrum resembles more that of a random coil conformation, and the polarization difference shows traces of structure, most likely arising from the small α-helical content of the protein. In Figure 3B, we show cross sections of the perpendicular polarization 2D-IR spectra for a pump frequency resonant with the high-frequency β-sheet mode (1688 cm^{-1}, indicated with a dashed line in Figure 2H and K) for increasing concentrations of Gdm^+. These cross sections show that both the diagonal peak and corresponding cross peak decrease significantly at moderate Gdm^+ concentrations and have completely vanished at a concentration of

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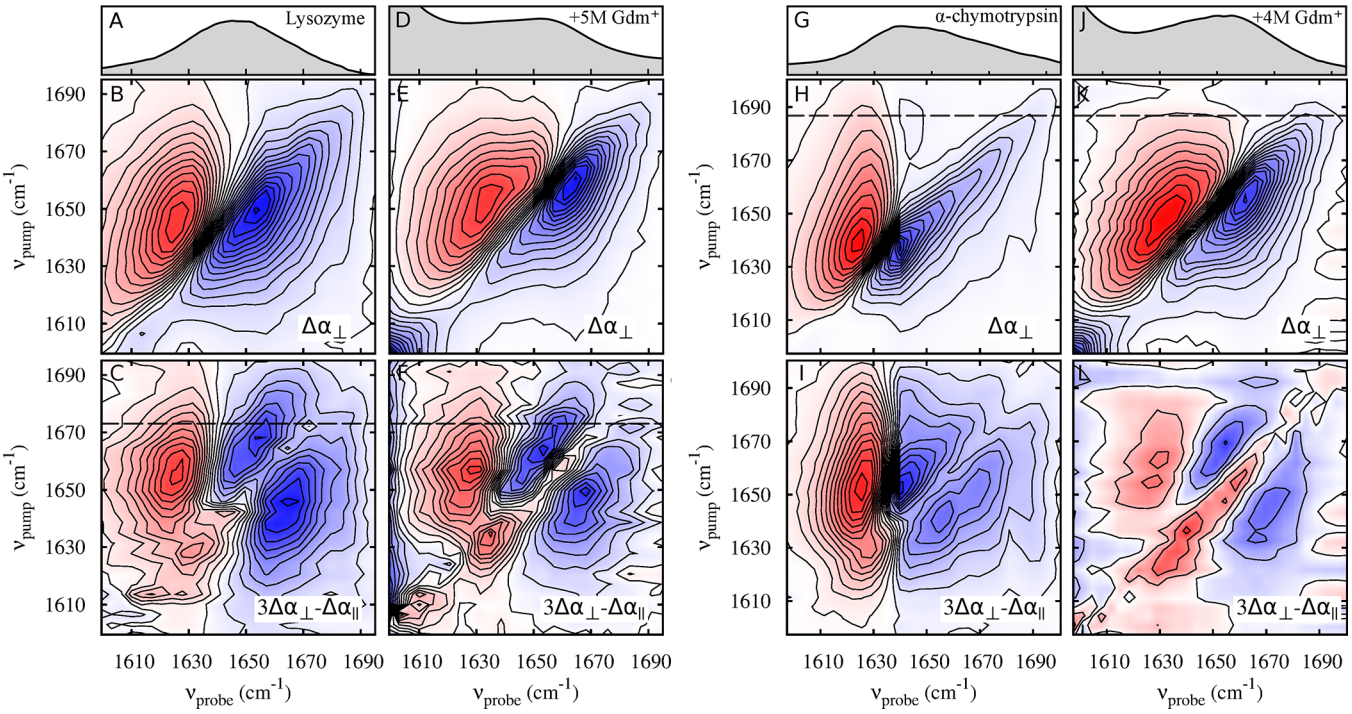

Figure 2. (A) FTIR spectrum of lysozyme (solvent subtracted). (B) 2D-IR spectrum of lysozyme for perpendicular polarization of pump and probe pulses; the contour intervals are 0.13 mO.D. Blue indicates a negative absorption change, and red indicates a positive absorption change. (C) Difference 2D-IR spectrum ($3\Delta\alpha_\perp - \Delta\alpha_\parallel$) of lysozyme; the contour intervals are 0.15 mO.D. (D) FTIR spectrum of lysozyme at a 5 M Gdm^+ concentration. (E,F) Corresponding perpendicular polarization and difference 2D-IR spectra; the contour intervals are 0.26 and 0.15mO.D, respectively. (G) FTIR spectrum of α-chymotrypsin (solvent subtracted). (H) 2D-IR spectrum of α-chymotrypsin for perpendicular polarization of pump and probe pulses; the contour intervals are 0.3 mO.D. (I) Difference 2D-IR spectrum of α-chymotrypsin; the contour intervals are 0.2 mO.D. (J) FTIR spectrum of α-chymotrypsin at 4 M Gdm^+ concentration. (K,L) Corresponding perpendicular polarization and difference 2D-IR spectra; the contour intervals are 0.1 and 0.2mO.D, respectively. The pump−probe delay of the 2D-IR spectra was 1.5 ps. The band at ~1610 cm^{-1} in (D) and (J) is the high-frequency wing of Gdm^+-$^{13}C^{15}N_3$ absorption. Complete data sets are shown in Figures S3 and S4 of the Supporting Information.

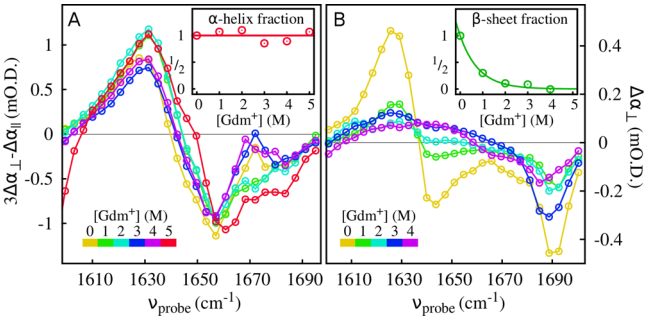

Figure 3. (A) Cross sections of the difference 2D-IR spectra of lysozyme for ν_{pump} = 1672 cm^{-1} at different Gdm^+ concentrations. (B) Cross sections of the perpendicular 2D-IR spectra of α-chymotrypsin for ν_{pump} = 1688 cm^{-1}. In the insets, fractions of the α-helical content of lysozyme and the β-sheet content of α-chymotrypsin as a function of Gdm^+ concentration are shown (lines are a guide to the eye).

4 M. In the inset of this figure, we plot the amplitude of the β-sheet cross peak as a function of Gdm^+ concentration, and a clear decay to zero is observed. This result indicates that the β-sheet secondary structure of α-chymotrypsin is completely destabilized by the presence of the Gdm^+ ions. This finding is consistent with the complete loss of enzymatic activity of α-chymotrypsin, which happens at a concentration of 3 M.[23]

Our results strongly suggest that Gdm^+ efficiently disrupts the tertiary structure of proteins as well as their β-sheet secondary structure but does not significantly destabilize the α-helical secondary structure. It should be noted that the increased sensitivity of 2D-IR spectroscopy (as compared to conventional IR spectroscopy) to the secondary protein structure was essential in reaching this conclusion. Our observations could be explained by a denaturation mechanism that would involve a strong decrease of the hydrophobic effect via direct interactions of Gdm^+ with the side chains of the proteins, thus promoting the exposure of hydrophobic core residues. α-Helices are often amphipatic,[24] and Gdm^+ seems to promote solvation of their hydrophobic side, disrupting in this manner the tertiary structure of the protein but leaving intact the α-helices themselves, probably because these are generally not significantly stabilized by hydrophobic interactions but rather by the intrinsic helical propensity of specific amino acids.[25] On the contrary, hydrophobic interactions between side chains promote the formation of β-sheets,[24] and we find that Gdm^+ destabilizes them very efficiently, most likely because it promotes solvation of their nonpolar groups, thus facilitating the detachment of the β-strands from each other. In addition to the hydrophobic effect, the difference in the hydrogen bonding topology between α-helices and β-sheets could make α-helices more difficult to chemically denature than β-sheets. In α-helices, each C═O group of residue i makes a hydrogen bond with the N−H of residue i + 4, except the first N−H groups and the final four C═O groups, which lack a hydrogen bond within the helix. However, it was found that numerous naturally occurring helices meet the condition of having flanking residues whose side chains can form these missing intrahelical hydrogen bonds,[26] resulting in more stable helices. This means that the majority of the residues inside of α-

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helices form helix-stabilizing hydrogen bonds. On the other hand, in β-sheets, hydrogen bonds are formed between neighboring β-strands; therefore, one-half of the edge strands of β-sheets are not involved in sheet hydrogen bond interactions. Hence, for a sheet consisting of two strands, only 50% of the residues will be forming hydrogen bonds that stabilize the β-sheet. For β-sheets with three strands, like most of the ones that constitute α-chymotrypsin, the number of participating residues increases to 60%, which is still much less than that in α-helices. This difference could partly account for the β-sheets of α-chymotrypsin being disrupted at low Gdm^+ concentrations as fewer hydrogen bonds keep the β-strands together after Gdm^+ has promoted solvation of the nonpolar side chains. However, for a larger number of β-strands, the percentage of hydrogen bonding residues increases, which might explain why larger β-sheets constructs, like amyloid fibrils, are more difficult to chemically denature with Gdm^+.[27]

To conclude, we find evidence that the α-helical structure remains in proteins after denaturation with Gdm^+, whereas β-sheets are easily destabilized. Residual structure in chemically denatured proteins is a controversial topic, especially because experimental probes of the secondary structure in such proteins are limited, but current experimental evidence indicates that the remaining structure (if any) is sequence-local rather than long-range.[28] Our study shows that 2D-IR spectroscopy provides a robust way of overcoming the challenges of investigating secondary structure in chemically denatured proteins, and our findings are in agreement with the existence of residual structure (either native or non-native) of local-sequence character.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods; additional FTIR spectra; 2D-IR spectra for parallel and perpendicular polarization of pump and probe pulses, and constructed difference spectra for all Gdm^+ concentrations of both lysozyme and α-chymotrypsin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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