# A Study of Urea-dependent Denaturation of β-Lactoglobulin by Principal Component Analysis and Two-dimensional Correlation Spectroscopy

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The water−urea−β-lactoglobulin interaction was studied by means of principal component analysis (PCA) and two-dimensional correlation spectroscopy applied to the urea concentration-dependent FTIR spectra of aqueous urea−protein solutions. The two $\nu$(C═O) and $\nu_{as}$(CN) bands coming from urea's absorbance, instead of the amide bands arising from protein, were employed in the analysis. To get a precise view of the changes induced by the urea concentration-controlled unfolding process, the absorbance variations developed in the ternary water−urea−protein system were compared with those observed in a binary water−urea system [Y.M. Jung et al., *J. Phys. Chem. B* **2004**, *108*, 13008]. The comparative studies enabled to detect apparent differences between the absorbance changes caused solely by urea's concentration increase and by the urea-dependent unfolding process. Urea's ability to unfold protein was discussed in context of the indirect and the direct mechanism depending on urea's concentration. It was shown that both mechanisms are relevant, that is, the indirect for solutions below 3 M and the direct for solutions above 3 M concentration. The character of the mechanism is strictly correlated with the association level of urea molecules.

## Introduction

The denaturation process of proteins by pressure,[1] temperature,[2−7] and chemical denaturants[6,8−20]are the subject of intensive study. A summary of what we have learned over the past 100 years about how urea denatures proteins can be found elsewhere.[21] Kamoun,[22] based on a pioneering paper by Nozaki and Tanford,[23] recalled that urea acts in large part by its effect on hydrophobic interaction. The denaturing action of urea on globular proteins causes stabilization of the unfolded form of a protein molecule both by diminishing the hydrophobic interaction between nonpolar groups and water and by increasing the affinity of the solvent for amide and peptide groups. On the basis of circular dichroism (CD) spectroscopy, Scholtz et al.[24] analyzed the interaction between urea and peptide groups as a major part of the denaturing action of urea on proteins. A more recent study of Muthuselvi et al.[25] shows that independently on a proposed mechanism a very high concentration of urea is required for denaturation of large biomolecules and that due to the association of urea with a protein−water system a hydrophobic interaction is enhanced.

With the development of modern experimental and calculation methods, more detailed facts about the denaturated function of urea were obtained. By means of molecular dynamics (MD) simulations, Caflisch and Karplus[26] revealed that urea denaturation involves effects on both nonpolar and polar groups of proteins molecules. It was shown that urea indeed interacts more favorably with nonpolar groups of the protein than water does, but in the presence of urea the interaction of water molecules with the hydrophilic groups of the protein is improved. Thus, urea denaturation involves effects on both nonpolar and polar groups of proteins. I was also found that urea interacts with the protein accumulating in the first solvation shell, causing the loss of secondary structure acting preferentially on the β-sheets while leaving the α-helices almost intact.[27,28] Moreover, it was suggested that urea and high temperature act through different unfolding mechanisms, revealing a differential sensitivity of protein secondary motives to various denaturant treatments.[28,29]

The MD simulations concerning the behavior of water and urea near the peptide suggest that urea acts indirectly in the denaturation process, by decreasing water mobility around the peptide, thereby increasing the ability to form peptide water hydrogen bonds with longer lifetimes, and also directly by giving urea molecules access to the polar groups of the peptide, thus providing better peptide solvation than pure water. Also, direct urea−protein interacts by preferentially forming hydrogen bonds to the peptide backbone, reducing the barrier for exposing protein residues to the solvent, and reaching the unfolded state was reported elsewhere.[30] In an excess of urea molecules in the solvation shell around the peptide, urea interacts very favorably with the peptide with very long residence times, and the water molecules in the shell get trapped between the urea molecules and the peptide, thus leading to reduced mobility.[29] Urea's ability to force a protein to unfold was analyzed by calculations of group transfer free energies (GTFEs).[31] It was postulated that interaction of urea with nonpolar side chains is not the favorable process that denatures protein, but that interaction of urea with the peptide backbone mainly drives the urea-induced protein unfolding, similarly to what was suggested by Tobi et al.[30] Because of the formation of hydrogen bonds between urea and the backbone groups, at high concentration of urea, oligomers of amyloidogenic forms can be destabilized.[32] Shimizu and Chan proposed a physical scenario applicable to molten-globule-like (MG) states[33] where the urea-denatured state conformations are not completely open but are relatively

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compact, with fluctuating and loosely formed hydrophobic contacts, most of which are partially exposed to solvent.[34]

Overview of literature concerning the experimental studies of urea−protein interaction done elsewhere[21,35] shows that the process more frequently has been studied by circular dichroism (CD), calorimetric, NMR, magnetic relaxation dispersion (MRD), and small-angle X-ray (SAXS) techniques. Infrared spectroscopy due to a strong limit caused by extensive overlapping of bands of ureas, waters, and proteins has not been such widely used for the investigations. However, some thorough studies have been done for ribonuclease A and staphylococcal nuclease,[3,8,12] but no such experimental data exists for $\beta$-lactoglobulin (BLG).

A first study on BLG denaturation by urea was done by optical rotation measurements in the late 60s.[36] Their authors postulated that it is a two-step process. The formation of the additional secondary structure was proposed as a first step, and the melting of all secondary structures was considered as the second. However, after thirty years of studies on the subject, it was stated on a base of CD and fluorescence studies that there is no evidence for the stable intermediate.[37,38] More recently, it was signaled by means of similar measurements[39] that urea induces one cooperative transition between native (N) and denaturated (D) states of BLG at pH 2.0 and 25 °C. Moreover, it was postulated that the secondary structure is more stable than the tertiary one during this transition, which confirms the molten globule-like state.[34]

The facts presented above show that the urea−protein interaction is a many-folded problem with the following uncertainties. Is it a direct or indirect mechanism? Which amide groups, polar or nonpolar, are mainly involved in the process? Which interaction, that is, urea−peptide backbone or urea−side chain groups is dominant? Is it a one- or two-level process in the case of BLG denaturation? Finally, it cannot be overlooked that all the events could depend on the concentration of urea.[40,41] The great ability of urea molecules to form dimers and oligomers has been revealed in many studies.[42−44]

The aim of our study is an analysis of the water−urea−$\beta$-lactoglobulin system as a function of urea concentration from 0.5 to 6 M by means of their FTIR spectra examined by principal component analysis (PCA) and two-dimensional (2D) correlation spectroscopy (2DCOS). To achieve a deeper insight into the three-components interactions, absorbance changes detected in the range of two bands of urea, that is, $\nu$(C═O) and $\nu_{as}$(CN), have been compared with the changes identified for the water−urea binary system measured under analogous conditions of urea concentration. Although the experimental settings excluded the possibility of direct analysis of the concentration-dependent denaturation profile of BLG by amide bands, comparative studies have been enabled on this discussion. In interpreting the spectral features detected for ternary systems by the PCA and 2DCOS methods, the fact of urea association and its influence on direct and indirect BLG−urea interaction have been taken into consideration.

## Experimental Section

$\beta$-Lactoglobulin and $C^{13}$-urea were purchased from Sigma Chemical Co. Ltd. and were used without further purification. Phosphate buffer (pH 6.6) solution was prepared with $D_2O$. For IR measurements, BLG and $C^{13}$-urea were deuterated, followed by lyophilization for the complete exchange of H to D. Deuterated $C^{13}$-urea solutions with different concentrations (0.5, 1.0, 1.5, 2.0, 3.0, 3.3, 3.4, 3.6, 3.8, 4.0, 5.0, and 6.0 M) were prepared in buffer solution. Deuterated BLG solutions (2 wt %) were finally prepared in the urea solutions with different concentrations. All BLG solutions were stored at room temperature for 36 h before the spectra were measured.

The IR spectra were measured at 2 $cm^{-1}$ resolution with a Bomem DA8 FTIR spectrometer equipped with a liquid nitrogen-cooled MCT detector. To ensure a high signal-to-noise ratio, 512 scans were coadded. A flow cell ($CaF_2$ window, Thermo Spectra-Tech. Inc.) was used in this study.

The necessary pretreatment procedures that have to be followed prior to the spectral variation for the $\nu$(C═O) and $\nu_{as}$(CN) bands that can be analyzed in a context of urea-denaturating properties are described in detail in our previous paper for the binary system.[43]

## Results and Discussion

Figure 1 shows, for the range from 1700 to 1300 $cm^{-1}$, the extent to which the bands for urea are more intensive than the amide bands and how strongly they are overlapped. Results after subtraction of the buffer spectral contribution from the ternary system are presented in Figure 1. The inset shows the results with subtraction done for the binary system. Also, the spectrum of BLG obtained for a urea-free system is plotted as a dashed line.

The main problem is that the band assigned to the amide I′ mode is located on a high-frequency wing of the urea band $\nu$(C═O), whereas the band of the second amide mode (amide II′) overlaps perfectly with the $\nu_{as}$(CN) band of urea. Moreover, the amide bands are drastically less intensive than the urea bands. We are challenged by the problem of how to eliminate from absorption of the urea−BLG mixture the spectral component that arises only from urea absorption. To do the subtraction quantitatively, as is necessary prior to the PCA and 2DCOS analyses, a region of solvent absorption that is not mixed with solute absorption has to be selected. However, here the subtraction cannot be controlled by absorbance assigned exclusively to urea absorption due to the strong overlapping. Thus, the traditional approach based on an analysis of absorbance changes attributed only to the amide bands has to be abandoned. We decided to analyze the urea-controlled denaturation of BLG by comparison between the spectra of the binary and the ternary system, subjected only to buffer subtraction.

**One-dimensional Analysis.** In a similar way to what was done for the water−urea system, it can be expected that the relative rearrangement between molecules of urea, water, and BLG should have an effect on the relative intensity of the $\nu$(C═O) and $\nu_{as}$(CN) bands, due to the varying polarity of the C═O and C−N bonds in the course of the denaturation process. As shown in Figure 2, where the absorbance ratios of the two bands for the binary and ternary systems are plotted, they are different from each other in the entire concentration range. This could not be caused only by the contribution to the analyzed values of the absorbance coming from BLG. This spectral component should simply lead to some constant shift between the two curves. Besides the shift, the two curves have different slopes in the range where urea molecules are linked both to dimeric units (below 2 M) and to larger oligomers (above 2 M). Simple analysis based only on comparison of absorbances for the two arbitrarily selected wavenumbers indicates that urea molecules in the ternary system are involved in interactions in environments of different polarity from what took place in the binary system. It can be expected that mainly the absorbance of the urea band assigned to the C═O group should be sensitive to varying interactions between urea−urea, urea−water, and urea−polar and urea−nonpolar sites of BLG.

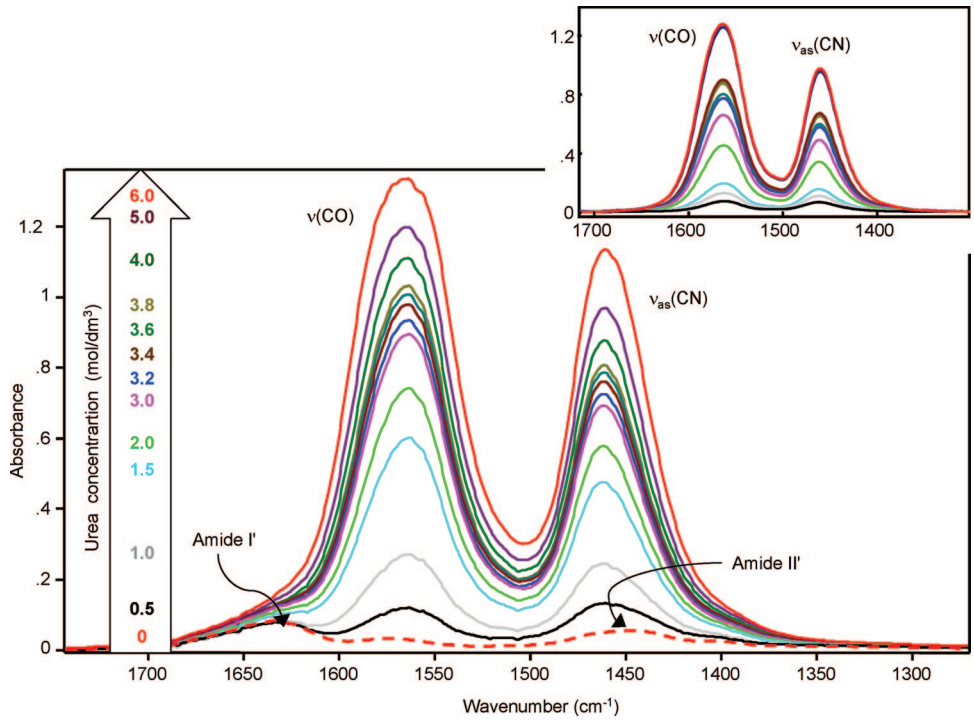


**Figure 1.** Absorbance evolution as a function of urea concentration in the range from 1700 to 1300 $cm^{-1}$ for the ternary system after subtracting the spectrum of buffer, baseline correction, and denosing procedure. The spectrum marked by the dashed line relates to the solution of BLG (2 wt %) without urea. The inset shows the same category of results for the binary system.

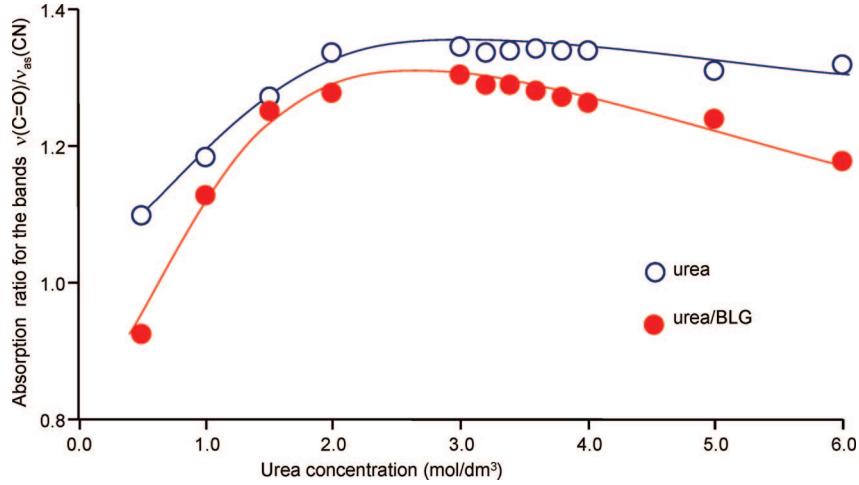


**Figure 2.** Ration of absorbance values taken at maximum for the two bands $\nu$(C═O) and $\nu_{as}$(CN) as a function of urea concentration for the system with (●) and without (○) BLG.

**The Step-to-step Difference Spectroscopy Analysis.** Frequently, an approach based on difference spectroscopy is employed for detailed analysis of spectral variations. Here, we propose a modified procedure that is based on calculation of difference between two adjacent spectra, that is, difference_spectrum($i$) = spectrum($i$) − spectrum($i$ − 1), instead of the commonly used method where the difference between an average spectrum and spectrum($i$) is calculated, where $i$ is the number of the sample. The step-to-step method permits examination of the magnitude of absorbance changes occurring between two closest experimental conditions. This should enable us to distinguish even small changes caused by a one-step perturbation increase. Before the difference spectra were calculated, their absorbance was normalized to unit concentration. Figure 3 presents the difference spectra as a 2D layout drawing. This shows that the largest changes occur at three initial steps of the perturbation. Moreover, with a concentration

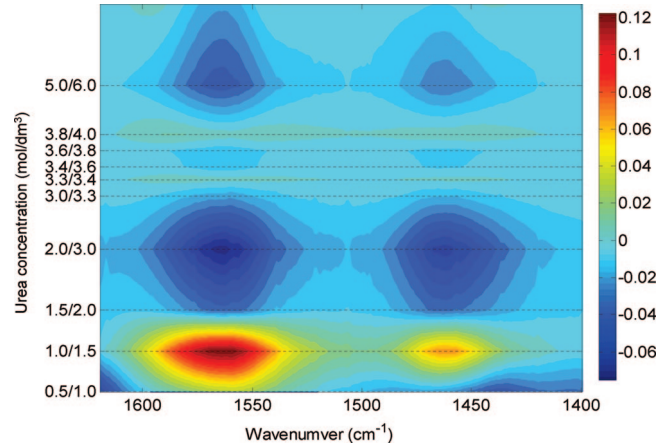


**Figure 3.** The difference spectra calculated in the step-to-step procedure for the ternary system presented in the 2D-mode.

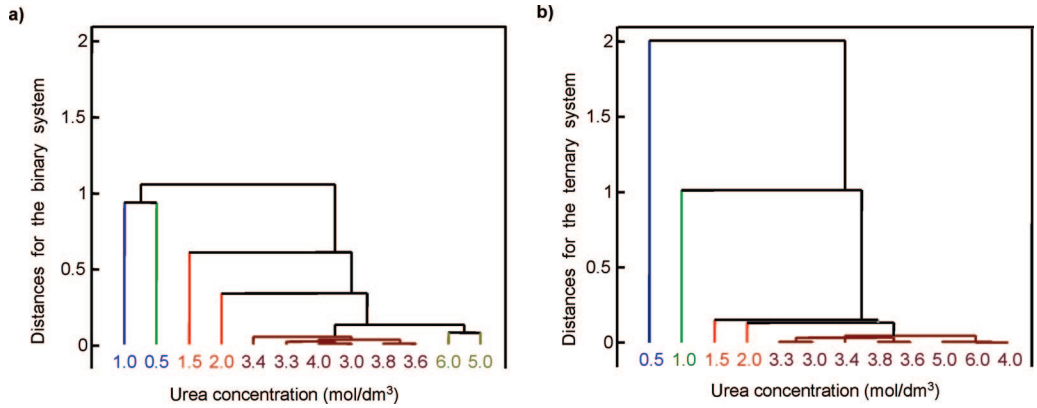


**Figure 4.** The classification of spectra for the system without (a) and with (b) BLG according to dendrogram plot.

increase the normalized absorbance also increases. Positive peaks are located around 1564 $cm^{-1}$; according to our studies for the binary system, this means that the changes should be assigned to urea molecules arranged in dimeric species. With a further concentration increase of urea, the spectral variations diminish and proceed in opposite directions. For systems between 3 and 4 M, the changes are stabilized at a level close to zero. An increase of urea concentration is again accompanied by an absorbance decrease; in addition, the changes are restricted to a narrower range of wavenumbers. Detailed analysis of the changes around 1560 $cm^{-1}$ reveals two subpeaks, at 1582 and 1552 $cm^{-1}$, assigned to C═O groups that are unbonded and bonded to oligomers, respectively. Similar studies done for the binary system (results not presented here) reveal that for higher concentrations the step-to-step changes are all positive. The absorbance for the $\nu$(C═O) and $\nu_{as}$(CN) bands was systematically increased. This fact means that, mainly for concentrations above 3 M, the urea-associated species are in different polar environments in the two systems. For concentrations below 3 M the step-to-step changes for the urea−$H_2O$ system are also positive. This could indicate the urea−water interaction as the dominant process in the presence of BLG molecules, that is, an indirect mechanism of BLG denaturation by urea dimers at concentrations below 3 M. Above this concentration the higher associates of urea show direct interactions with the side chains of BLG.

**Dendrogram Analysis.** To test differences of the concentration-dependent evolution of absorbance for the $\nu$(C═O) and $\nu_{as}$(CN) bands for the binary and the ternary system, an analysis based on a dendrogram plot that provides a two-dimensional (2D) pictorial representation of the clustering process was also performed. The graphic form simplifies and demonstrates the structure within the data and clearly illustrates the similarity between the different samples.[45] This approach allows the spectra to be classified independently according to their similarities for the two series. In Figure 4, the two dendrograms are presented at the same scale in the *Y* direction, i.e., distance between samples. For the system without BLG, mainly for urea concentrations less than 3 M, the similarity between the samples is larger.

Above the 3 M concentration, the spectra of the two sets are characterized by comparable distances of similarities; however, they are differently clustered. The dendrogram for the system without BLG offers a quantitative evaluation of the influence of the urea association process on the absorbance changes in the analyzed frequency range. It shows that dimerization of urea causes almost five times larger spectral variations than formation of oligomeric chains developed at a concentration above 2 M.

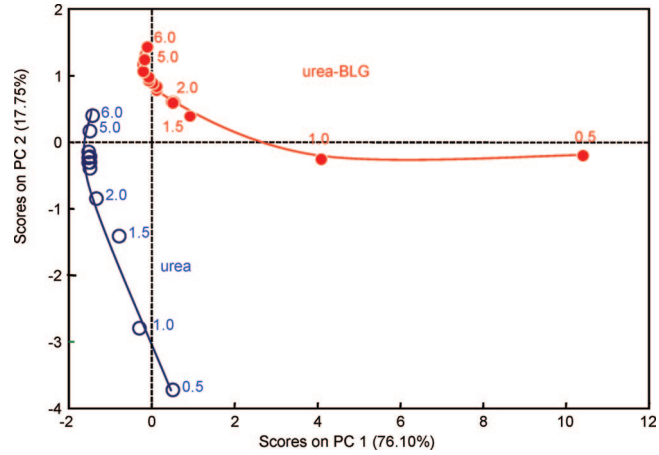


**Figure 5.** Scores plot of the spectra for the system with (●) and without (○) BLG combined into one matrix on the first two principal components.

For the system with BLG, the dendrogram reveals different relative magnitudes of absorbance changes caused by urea concentration changes. There is a big discrepancy between the 0.5 and 1 M solutions and all more highly concentrated samples. Moreover, the solutions above 4 M are much more similar to each other than to the system without protein. The differences cannot be unambiguously interpreted, as they can be caused by two effects that cannot be separated. First, they could indicate that, although the bands for BLG are strongly overlapped by the bands from urea, their presence causes the changes for the three-component system to be different from those for the two-component one. This suggests that by employing such methods as 2DCOS and/or PCA it should be possible to obtain direct information on the spectral changes induced by the urea-controlled denaturation of BLG. However, there could be other source of the differences. It cannot be ruled out that its two bands may be modified due to the direct or indirect denaturation of BLG by urea. In a further detailed analysis based on the PCA and 2DCOS methods, the two possibilities will be considered.

**The Principal Component Analysis.** In this analysis, the two sets of data were combined into one matrix that was next used for PCA. Prior to the PCA calculations, the data matrix composed of 24 rows (2 × 12 samples) and 468 columns was subjected to standard normal variate (SNV) normalization that scales rows of the data matrix to mean zero and unit standard deviation. Next, a mean-centering procedure was implemented to scale the data matrix to mean zero. Figure 5 shows the scores values for the two sets against PC1 and PC2. The first component describes more than 76% of the total absorbance

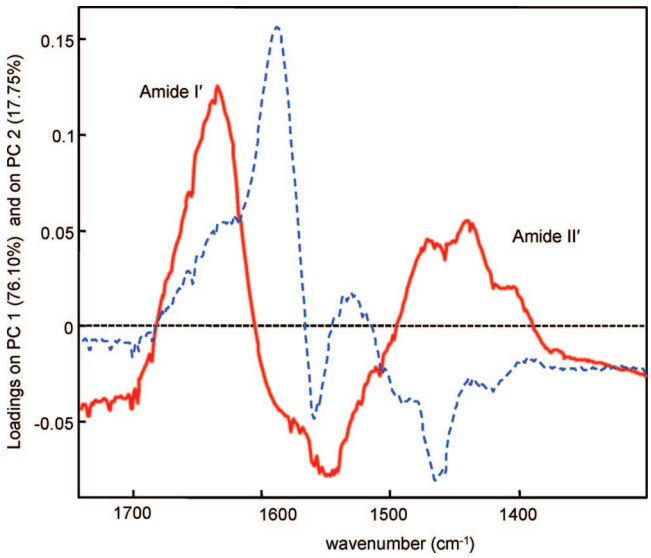


**Figure 6.** Loadings plot of the spectra for the two systems combined into one matrix for the PC1 (solid line) and PC2 (dashed line).

changes held by the data matrix, whereas the second component captures almost 18% of the remaining variances not described by PC1. The two sets are clearly separated over the two components. The changes attributed to the ternary system are mainly captured by PC1, but over PC2 grouped changes are developed for the binary system. It confirms that BLG−urea interaction enhances the absorbance changes in the analyzed range by more than four times. The loadings plot presented in Figure 6 enables us to classify the absorbance changes into ranges attributed to the two systems. Using the PCA approach, it was possible to detect the changes that are directly correlated with the amide I′ and amide II′ bands. These are indicated the two positive PC1 peaks around 1634 and 1440 $cm^{-1}$, respectively. The values are in good agreement with the literature data, where assignments for the two bands for different proteins are presented.[46] Negative peaks around 1548 $cm^{-1}$ characterize the solutions above 3 M and should be assigned to urea's species associated with forms larger than dimers. A further negative peak around 1560 $cm^{-1}$ can be seen; according to our assignment,[43] this is a signature of the dimeric association of urea. The strong positive peaks at 1588 $cm^{-1}$ for PC2 indicates the presence of strong changes that should be attributed to the free C═O groups. As this peak is dominant on the PC2 loadings plot, it means that the two systems are separated by the second component, due to the differences in absorbance attributed to the free C═O groups. This suggests that the influence of the medium surrounding these groups on their absorbance has to be different for the two systems. Certainly, the modification of the environment for the free C═O groups is much larger for direct than for indirect interaction. On the basis of the PCA results, the following scenario can be proposed for the interaction. For solutions above 3 M, direct interaction between BLG and urea is responsible for the denaturation properties. The urea species approaching molecules of BLG stabilize their molten-globule-like state by coating the expanded chain of BLG. In the coating layer, the free C═O groups find locations in the vicinity of nonpolar side chains.

**The 2D Correlation Analysis.** A very good possibility for comparison of the absorbance changes for the two systems is given by 2D correlation spectroscopy (2DCOS) in the homo- and heterosample mode.[47] Recently, Iloro et al.[48] used the 2DCOS to monitor the urea- and heat-induced unfolding denaturation of sarcoplasmic reticulum $Ca^{2+}$-ATPase, as it is

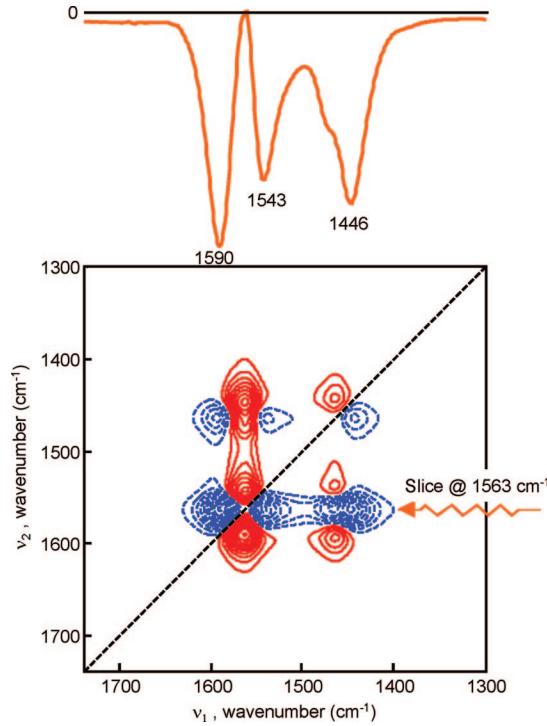


**Figure 7.** Homosample asynchronous 2D correlation spectrum for the ternary system along with the slice spectrum extracted at position of its maximal asynchronicity presented on a top. Solid and dashed lines represent positive and negative cross peaks, respectively.

mainly the asynchronous spectra that shed a new light on the phenomena studied here the synchronous spectra that were also calculated are omitted. In addition to the results for the heteromode analysis, those obtained in homo mode also seem to be interesting. Figure 7 shows the asynchronous spectrum calculated for the ternary system only. To facilitate the detection of the 2D peaks, a slice spectrum at 1563 $cm^{-1}$ is also demonstrated in Figure 7. There are two very noticeable asynchronous peaks in a range of the $\nu$(C═O) vibration, that is, at (1590, 1563) $cm^{-1}$ and at (1563, 1543) $cm^{-1}$. By applying classical rules to their analysis[49] it was possible to make the following assignment of the order of absorbance changes induced by the urea-controlled denaturation of BLG:

Order of absorbance changes from earliest to latest.

$1563\ cm^{-1}$ $1543\ cm^{-1}$ ≈ $1590\ cm^{-1}$

Increase of urea concentration from 0.5 M up to 6 M

First, as was already postulated, the process starts from direct water−BLG interaction that affects the urea dimers interaction, leading to the variations at 1563 $cm^{-1}$. Direct contact of dimers with water molecules leads to weakening of the water interaction with BLG and to reduction of the hydrophobic forces keeping the BLG molecules in a native state. It is a classical example where the protein structure is mediated by water molecules.[50]

At the next stage, which starts for solutions above 3 M, the denaturation mechanism changes into the direct one. The expanded structure of BLG after weakening of the hydrophobic forces becomes opened on the direct interaction with the associated urea species, which contain a majority of bonded, but also some nonbonded, C═O groups. The two groups experience different influence from the polar and nonpolar fragments of the BLG chain. In comparison with their interaction

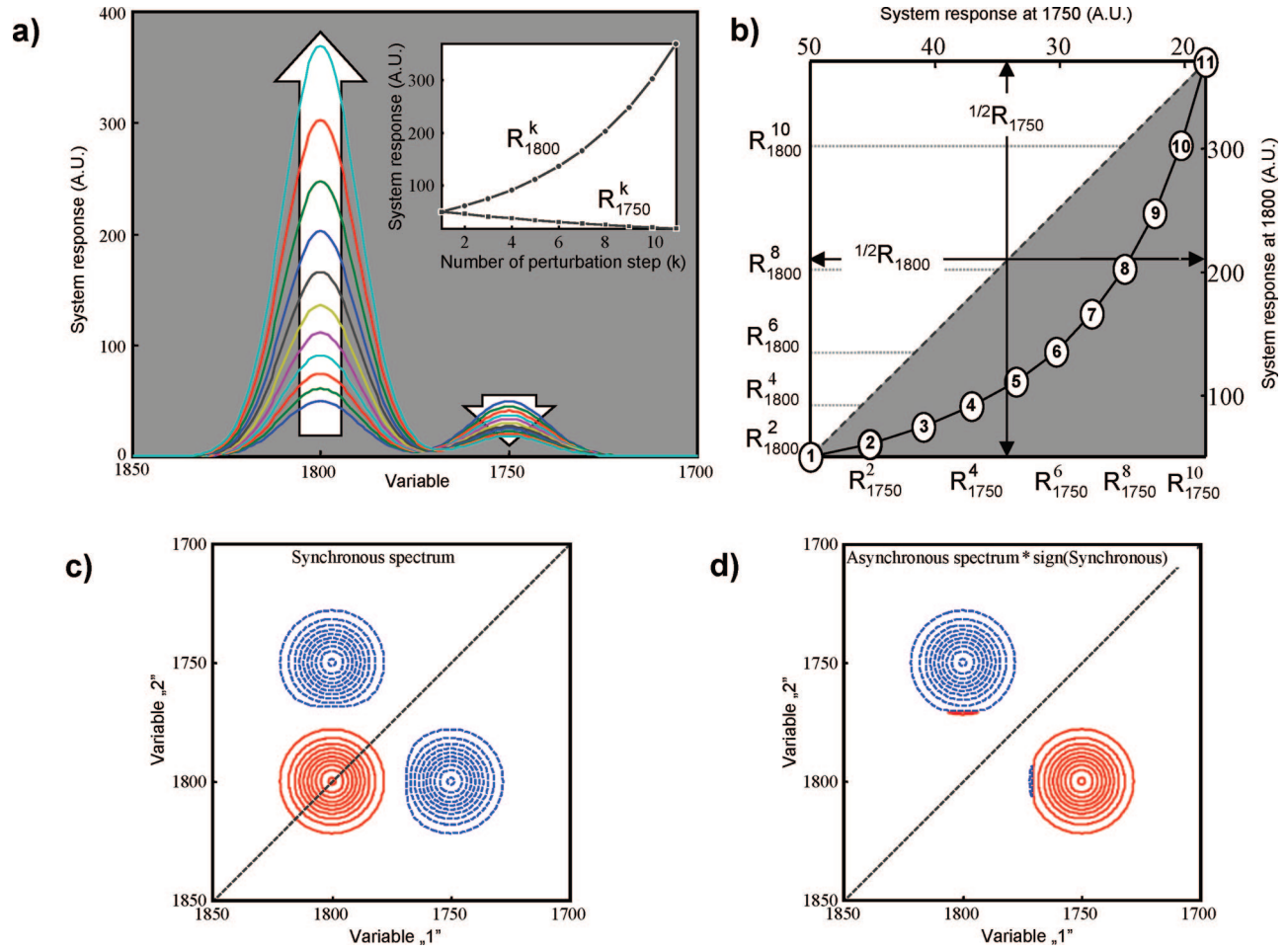


**Figure 8.** Simulated spectra (a), the variable space plot (b), the synchronous (c), and the asynchronous (d) 2D correlation spectra calculated for the simulated data. Solid and dashed lines represent positive and negative cross peaks, respectively.

in the binary system, the urea associates are transferred to a more heterogeneous environment in the context of local dielectric properties. The changes are manifested both by the bonded (variations at 1543 $cm^{-1}$) and by the nonbonded (variations at 1590 $cm^{-1}$) C═O groups.

Although it is beyond the scope of this paper, we would like to relate to a recently published paper[51] that addresses a critical analysis of Noda's rules. The paper could cause major confusion, mainly for nonexperienced users of the 2D analysis. Whenever some advanced method of analysis is employed, first of all, the data should be explored by ordinary methods. Mainly, for two well-separated peaks, an analysis based on a variable space plot proposed by Isaksson et al.[52] is strongly recommended. Figure 8a presents the simulated data composed from two peaks located at variables 1800 and 1750 that intensities are changing in the forms $y(x) = 40.94 \exp(0.2x)$ and $y(x) = 55.26 \exp(-0.1x)$, respectively. The data specifically match to Huang's data, presented in Figure 4.[51] In light of his results, there is a conflict in the rate of the changes estimated from the signs of synchronous and asynchronous cross peaks and from properties of the two exponential functions. First, the sequential order of events occurring during the observation period, which is not the same as the rate of changes, should not be drawn, from unscaled *y*-values plotted against the perturbation step, as it is on the inset of Figure 8a. The *y*-values arranged in an increasing direction of perturbation step should be explored in a variable space plot, as shown Figure 8b. A diagonal line is added to the plot that axes limits are scaled to the range of system response at 1750 and 1800. Whenever the points marked in Figure 8b by a circle, with an index of perturbation step inside, are below the diagonal line, the changes plotted on the *X*-axis are ahead of these plotted on the *Y*-axis. For points located above the diagonal line the rule is inverted. A distribution of points in Figure 8b reveals that the intensity changes at 1750 occurs before these at 1800. Exactly the same order is determined by the signs of synchronous and asynchronous cross peaks, as is shown in Figure 8, panels c and d. For all cases simulated by Huang, complete agreement between conclusions determined from the variable space plot and from Noda's rules was achieved. Also, an inspection of perturbation steps at which the variations reach midpoint ($^{1/2}R_{1750/1800}$) shows that for the peak at 1750 the point is close to fifth step, but for the peak at 1800 this point is at least three steps after that. The above-described method can be easily applied to separated peaks, but more frequently an experimental data are composed from many strongly overlapped contributions. In that case, Noda's rules, undermined by Huang's false results, allow the establisment of the sequence of real-world changes.

Coming back to the problem of denaturation of BLG in urea's aqueous solution, a further fact emerges from the 2D hetero-sample analysis. Figure 9 shows the asynchronous spectrum, where the horizontal axis relates to the ternary and the vertical axis to the binary systems. The most important feature seen in this figure concerns the absorbance changes observed in the two systems around 1560 $cm^{-1}$. The changes come mainly from the dimers that play a crucial role in the indirect urea−BLG interaction. The sign of the peak means that the changes in the ternary systems are ahead of those in the binary system. It

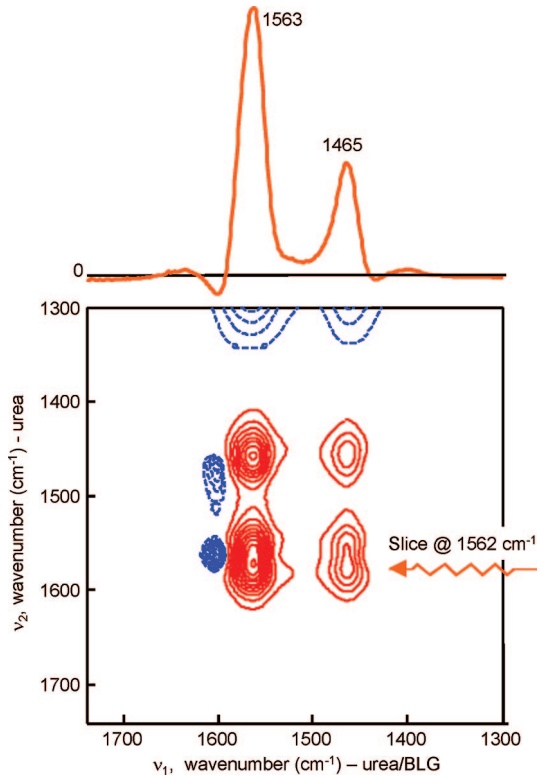


**Figure 9.** Heterosample asynchronous 2D correlation spectrum for the two systems along with the slice spectrum extracted at position of their maximal asynchronicity presented on a top. Solid and dashed lines represent positive and negative cross peaks, respectively.

reveals that in the presence of BLG molecules the urea−urea and urea−water interactions become more sensitive to changes in urea concentration. Although in the direct mechanism the BLG−urea straight contacts are neglected, the interaction of water molecules with polar groups of BLG that form the hydrophilic surface of BLG in native form has a significant influence on the structure of the urea dimers. Evidence that water molecules are tightly associated with urea, forming specific urea−water complexes, has recently been provided by a polarization-resolved mid-infrared pump−probe spectroscopy study.[44] Because of the water−BLG interaction, accompanying urea dimers become less stable and therefore more sensitive to an increase of urea concentration changes, leading to the picture shown in Figure 9.

## Conclusion

The urea concentration-dependent FTIR spectra of aqueous urea−protein solutions allowed us to study the ability of urea molecules to drive protein into a denaturated state. The analysis was based on the two $\nu$(C═O) and $\nu_{as}$(CN) bands corresponding to absorbance of urea. First, by means of ordinary 1D analysis and, recently proposed, the step-to-step difference spectroscopy analysis, it was determined that, due to the presence of $\beta$-lactoglobulin, the concentration-dependent evolution of the bands is different from that observed for system free of the protein. A comparative analysis of the differences done by means of PCA and 2DCOS provided details on the interaction of urea molecules with the main- and the side-chain amide groups. It was shown that a molecular description of how protein is denaturated by urea molecules should be strictly correlated with the association of urea molecules. Depending on urea's concentration, the association process proceeds in two stages and is characterized by different types of urea associates. Obtained results have also shown that the denaturation process should be considered as a phenomenon that is dependent on urea's concentration. It reveals that a balance between the indirect and direct mechanism of protein unfolding has to be considered in context of the association profile of urea. It can be postulated that in a wide range of urea concentration both mechanisms are probable. However, the indirect mechanism, when urea acts directly by binding to the polypeptide, dominates for solutions below 3 M, whereas for solutions above 3 M concentration the scenario where urea acts indirectly on the protein by perturbing the water-mediated interaction is more probable.

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