

# Urea as a protein destabilizing agent in electrospray ionisation

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**Urea is well known as a denaturant of proteins, but there is also evidence that millimolar amounts of urea may in fact stabilize protein complexes. Advances in mass spectrometric analysis have given us the opportunity to test the effect of urea on several noncovalent complexes in buffered solutions. We expected to see lower charge states if folded proteins were more compact (and therefore more stable), and higher charge states if the proteins were denatured. We have found that mM urea interferes with some noncovalent interactions, and that the extent of interference depends on the specific protein complex. The difference seems to be related to the type of interactions, with weak ones, such as H-bonds, more sensitive to urea. Examples show that a quick check with urea may give some insights into protein stability in the mass spectrometer. Copyright © 2009 John Wiley & Sons, Ltd.**

Electrospray and nanospray ionization can be used to study large noncovalent complexes because the gentleness of ionization conserves hydrophobic and ionic interactions. Under the right spray conditions, even fragile complexes can survive transition to the gas phase, and adjustments to those conditions can give valuable information about the stability of the complexes. One critical phase of this transition is the desolvation step where excess water and buffer are dried off the ion, leaving the charge on a 'bare' protein.<sup>1</sup> Simulations of the desolvation process confirm the validity of mass spectrometry measurements for the determination of protein structure.<sup>2</sup> Inefficient desolvation produces ions with water and buffer adducts, and thus poor resolution. On the other hand, conditions that are too harsh can cause disruption of the noncovalent interactions.

For small proteins, it has been shown that charge state depends on the combined gas-phase basicities of the buffer components and the basic side chains of the surface amino acids.<sup>3</sup> A number of factors, such as addition of acid or higher voltage conditions in the mass spectrometer,<sup>4</sup> or treatment with nitrobenzyl alcohol,<sup>5</sup> can produce an increase in the charge state of a given protein. It is generally accepted that folded structures have lower charge states than do unfolded proteins, possibly because of lower solvent accessibility,<sup>6</sup> but solvent surface tension has no apparent effect on the charge-state distribution.<sup>7,8</sup> Catalina *et al.*<sup>9</sup> have demonstrated that the charge state of some folded proteins can be further reduced by addition of a strong organic base such as 1,5-diazabicyclo[4.3.0]non-5-ene (DBN). This and other 'proton

sponges' remain basic in the gas phase and cause a considerable loss of charge without denaturing most proteins. However, these compounds are very difficult to use in the mass spectrometer,<sup>9</sup> and their biological relevance is unknown.

Early crystallographic work suggested that urea stabilizes the flexible parts of folded proteins, and relaxes the crystal packing contacts.<sup>10</sup> Bhuyan<sup>11</sup> has also shown, using different methods, that low (mM) concentrations of urea may stabilize proteins. The thermal dissociation of CO from ferrocyanide was slowed at low 'salting-in' concentrations of urea or guanidine hydrochloride. However, at higher concentrations of the denaturants the dissociation was more rapid and further enhanced by the presence of NaCl. If a low concentration of urea stabilizes parts of a protein's structure, one might expect a more compact structure, and thus a lower charge state. On the other hand, if increasing amounts of urea lead to destabilization, we might expect a higher charge state as the protein unfolds.

We have found that 10 mM urea is quite acceptable as a buffer component for both electrospray and nanospray ionization, but it has variable effects that differ with the type of noncovalent complex being analyzed. Thus, urea can be used to determine information about the stability and ionic interactions of several large noncovalent complexes, as shown below.

## EXPERIMENTAL

### Preparation of samples

Yeast alcohol dehydrogenase (ADH) and jack bean conalbumin A (ConA) were purchased from Sigma (St. Louis, MO, USA), and used without further purification. Citrate synthase (CS) was prepared from the cloned gene expressed

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in *Escherichia coli*, and purified as previously described.<sup>12,13</sup> *E. coli* catalase HPII was purified from transformed *E. coli* strain UM255.<sup>14</sup>

Ammonium bicarbonate buffer and urea solutions were each passed through a 0.2  $\mu\text{m}$  syringe filter before use. Ammonium acetate (99.99%, Aldrich) was used directly at the appropriate concentration. DBN (1,5-diazabicyclo [4.3.0]non-5-ene, 98%, Aldrich) was prepared at 0.1 M in water, and then adjusted to pH 7 with acetic acid.<sup>9</sup>

Each of the pure proteins was changed into the chosen buffer using a Centricon<sup>TM</sup> (Amicon) ultrafiltration device with appropriate mass cut-off.<sup>15</sup> Samples, with appropriate negative controls, were incubated with buffered urea or DBN for 4 h at room temperature before mass spectrometric analysis. Longer incubation times were not used because the spectral quality decreases the longer samples are stored in ammonium-based buffers. There was some drift to the pH during the 4 h incubation: all the buffers except ammonium acetate with urea increased by about 0.3 pH units.

### Mass spectrometry

The proteins were analyzed on a new time-of-flight instrument constructed at the University of Manitoba, mostly from Sciex prototype parts.<sup>16</sup> It is similar to the mass spectrometer used previously for our noncovalent measurements,<sup>17</sup> but it has 16 kV accelerating voltage instead of 4 kV. Thus, both transmission and detection efficiency are expected to be considerably higher for large ions. For nanospray ionization, a 2  $\mu\text{L}$  aliquot of pure protein at 1  $\mu\text{M}$  concentration was introduced into a nanospray capillary held in a custom-made holder. A small amount of back pressure was applied via a hand pump until the spray was stabilized. The nanospray voltage was adjusted to give the best resolution with minimum disturbance to the protein; details for each protein are given in the appropriate figure caption. For electrospray ionization, a 'Genie' pump (Kent) fitted with a 25  $\mu\text{L}$  Hamilton syringe was used to spray from a 26-gauge metal capillary at 0.2  $\mu\text{L}/\text{min}$ .

### Circular dichroism (CD) of citrate synthase

CD spectra were acquired with a Jasco 810 spectropolarimeter/fluorometer using samples prepared in 20 mM Tris-Cl, 1 mM EDTA, pH 7.8. Protein concentrations were determined using the molar absorptivity of 47699  $\text{M}^{-1}\text{cm}^{-1}$  at 278 nm.<sup>18</sup> Protein and urea (0 to 2 M) were incubated overnight at room temperature. For acquisition of near-UV spectra we used 17  $\mu\text{M}$  protein in a quartz cuvette with a 1.0 cm path length. Spectra were collected at 50 nm/min between 340–250 nm with a response time of 4 s and data pitch of 0.1 nm. For acquisition of spectra from the far-UV, we used 1.8  $\mu\text{M}$  protein in a quartz cuvette with a 0.1 cm path length. Spectra were collected at 20 nm/min between 250–210 nm with a response time of 4 s and data pitch of 0.1 nm. For each experiment, baselines were collected from the appropriate buffer solution, and spectra were baseline-corrected. The CD intensity and wavelength of the spectropolarimeter were calibrated using solutions of d-10-camphorsulphonic acid.<sup>19</sup>

### Activity measurements of citrate synthase

Citrate synthase in 20 mM Tris, 1 mM EDTA, pH 7.8, was incubated with 0–2 M urea and incubated at room temperature for 0, 2, 4 or 24 h. Enzyme activity was measured with 50  $\mu\text{M}$  acetyl coenzyme A, 100  $\mu\text{M}$  oxaloacetic acid, 100  $\mu\text{M}$  5,5-dithio-*bis*-(2-nitrobenzoic acid).<sup>20</sup>

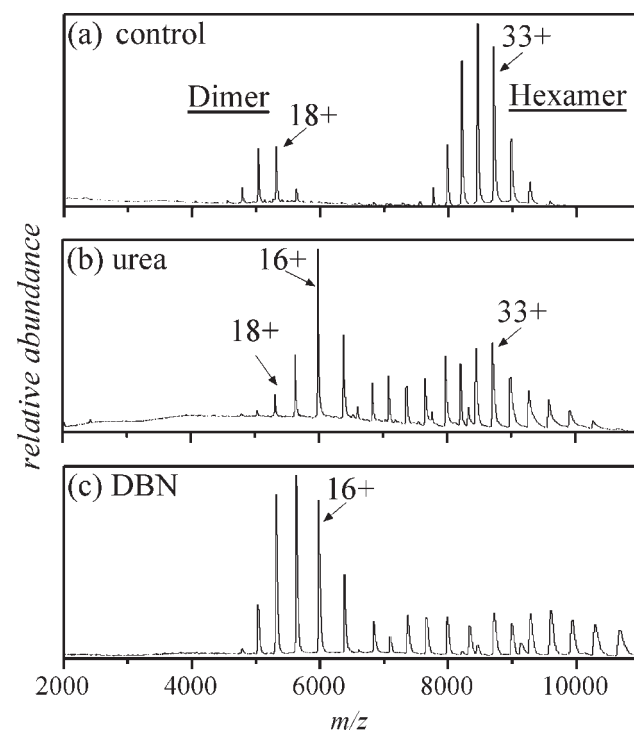
### Size-exclusion chromatography (SEC) of CS

A Sephadex G200 (Pharmacia) column (3  $\times$  40 cm) was equilibrated with 20 mM Tris-Cl, 1 mM EDTA, pH 7.8;  $V_0$  was determined with Dextran Blue before running each of the protein samples. A 1 mL sample of pure CS (2 mg) and 5% glycerol was applied to the column and eluted in the same buffer in 50 mL fractions. The experiment was then repeated using 1 M urea in both column and protein sample. Presence of CS was determined by absorbance at 278 nm and by activity measurements.

## RESULTS

### *E. coli* citrate synthase

Citrate synthase (CS) has a subunit mass of 47885 Da, and shows an equilibrium mixture of dimers and hexamers in the mass spectrometer.<sup>13</sup> This equilibrium is affected by the concentration of the buffer (which mimics KCl, an activator) and by NADH (the allosteric inhibitor).<sup>13,21</sup> The protein has been shown to undergo a two-step unfolding in response to urea, with a stable intermediate between 2.5 and 5.5 M urea.<sup>22</sup> However, low (mM) urea has no effect on activity.<sup>18</sup> Nanospray ionization from 20 mM  $\text{NH}_4\text{HCO}_3$  without urea shows two well-separated charge envelopes (Fig. 1(a)). The envelope of the 96 kDa dimer (near  $m/z$  5000) has four distinct



**Figure 1.** Nanospray spectra of 1  $\mu\text{M}$  *E. coli* citrate synthase prepared in 20 mM ammonium bicarbonate. New Objective<sup>TM</sup> PicoTip, 280 V focus, 1 kV spray: (a) control; (b) 4 h incubation with 10 mM urea; and (c) 4 h incubation with 2 mM DBN.

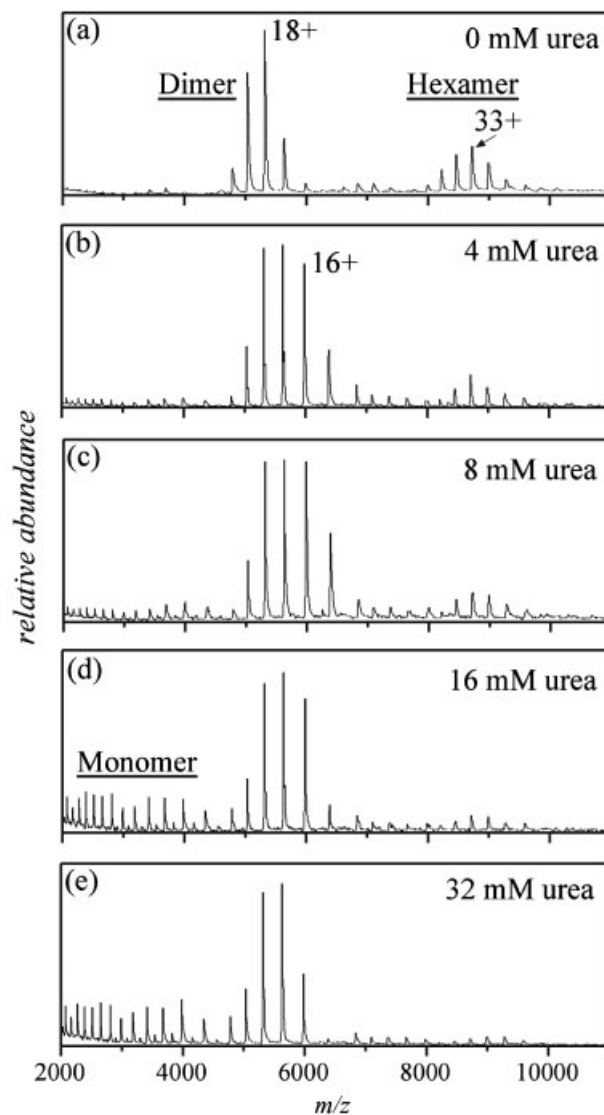
ions, with 18+ the most abundant, and the envelope of the 288 kDa hexamer (near  $m/z$  9000) has seven ions, with 34+ the most abundant. The spectra of samples incubated with urea show major differences, both in the relative charge on the ions and in the relative abundance of dimer and hexamer ions. By 10 mM urea (Fig. 1(b)) the dimer charge envelope is now centred at 16+, the envelope for hexamer ions is broader and less distinct, and there is some evidence of ions from the tetramer in the region near  $m/z$  7000. There is no measurable mass increase on any conformation. Addition of DBN at 2 mM produces a spectrum somewhat similar to that with 10 mM urea, but ions from the hexamer are less abundant (Fig. 1(c)). There is no evidence of ions from the monomer.

In order to minimize the effect of buffer ions,<sup>3</sup> spectra were acquired using electrospray ionization with a lower concentration (5 mM) of  $\text{NH}_4\text{HCO}_3$ .<sup>13</sup> The lower concentration of ammonium salt in the buffer changes the equilibrium to one with more dimer ions,<sup>21</sup> but the peak charge states on the dimer and hexamer are the same as seen in 20 mM  $\text{NH}_4\text{HCO}_3$  (cf. Figs. 1(a) and 2(a)). Figure 2 shows a titration with constant spray conditions and increasing concentration of urea. Addition of urea has three effects on the charge distributions of the ions; by 4 mM urea there is loss of net charge on the ions of the dimer, decrease in the abundance of ions of the hexamer, and appearance of ions of the monomer. By 32 mM urea, ions from the hexamer have all but disappeared, with a concomitant increase in ions from the monomer. At low  $m/z$  (not shown) there is considerable 'noise' which increases with the urea concentration. The loss of charge on the dimer and hexamer ions (particularly noticeable in the nanospray experiments) suggests a more compact protein with less surface area, but the appearance of monomer ions in the harsher, electrospray experiments denotes denaturation.

We checked to see if standard biochemical measurements would cast any light on this behavior; CD measurements would measure loss of helicity, assays would measure loss of activity, and SEC would tell us if the protein was a dimer or a hexamer when in millimolar urea. None of the CD measurements showed any difference between control and protein solutions containing up to 1 M urea. By 2 M urea there was a 25% loss of intensity in the near-UV measurements. At low concentration of urea (<50 mM) there was no loss of activity, even after 24 h incubation, although higher concentrations of urea did produce a slight loss of activity, as shown earlier.<sup>18</sup> SEC on Sephadex G200 showed no difference in elution volume, whether the buffer was Tris-EDTA or the same buffer with 1 M urea, consistent with the hexamer conformation (data not shown). These experiments suggest that the effect of low concentration of urea on CS is specific to events in the mass spectrometer, where the protein concentration is much lower than in most conventional biochemical measurements. It was therefore of interest to ascertain whether the same could be said of other proteins.

### Other proteins

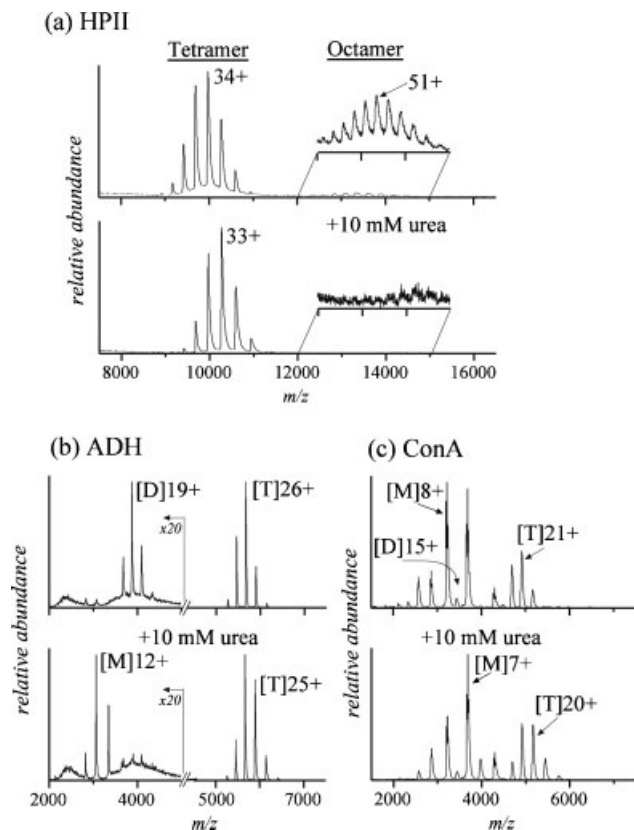
Catalase HPII from *E. coli* has a subunit mass of 84035 Da, but the active protein is a tetramer with four associated heme molecules and a total mass of 339100 Da.<sup>23,24</sup> Under gentle ionization conditions clusters of the tetramer have been



**Figure 2.** Electrospray spectra of 5  $\mu\text{M}$  *E. coli* citrate synthase prepared in 5 mM ammonium bicarbonate. Metal capillary with 280 V focus, 3 kV spray: (a–e) 4 h incubation with urea as shown on spectrum.

observed, but extreme voltage is required to remove the heme.<sup>23</sup> As shown in Fig. 3(a), the native protein has two charge envelopes, tetramer ions centred at 34+, and a small amount of octamer ions centred at 51+. Incubation with 10 mM urea causes a slight decrease of the relative charge on the tetramer ions and complete loss of the octamer ion envelope. There is neither loss of heme, nor appearance of any species smaller than tetramer.

Alcohol dehydrogenase (ADH) from yeast prepared in ammonium acetate is a stable tetramer of  $147523 \pm 40$  Da, although other work suggests a monomer-tetramer equilibrium.<sup>25–28</sup> Our spectrum for ADH (Fig. 3(b)) is similar to that of Potier *et al.*,<sup>25</sup> with a major charge envelope of 25+ to 27+ representing tetramer ions (147766 Da), but, in addition, we observe a small envelope of ions from a dimer (73860 Da), not previously reported. Our spray conditions may be gentler than those used by others<sup>27,28</sup> so that the dimer can survive transit through the desolvation stage. Addition of low concentrations of urea causes a slight loss of charge on the ions of the tetramer, so that now the 25+ ion is the most



**Figure 3.** (a) Nanospray spectrum of  $1\ \mu\text{M}$  *E. coli* catalase HPII prepared in 10 mM ammonium acetate. New Objective™ PicoTip, 280 V focus, 1 kV spray. (b) Nanospray spectrum of  $1\ \mu\text{M}$  yeast alcohol dehydrogenase prepared in 10 mM ammonium acetate. New Objective™ PicoTip, 180 V focus, 1 kV spray. The left part of the spectrum, from  $m/z$  2000 to 4500, is 20 $\times$  that of the higher  $m/z$  region. (c) Nanospray spectrum of  $1\ \mu\text{M}$  jack bean concanavalin A prepared in 10 mM ammonium acetate. New Objective™ PicoTip, 180 V focus, 1 kV spray. In each pair of spectra, the upper one is the control, the lower one after 4 h incubation with 10 mM urea. [M] = monomer, [D] = dimer, [T] = tetramer ion.

abundant. However, the most striking effect is the loss of dimer ions and appearance of ions from the monomer. Deconvolution of this new monomer envelope gives two peaks, of masses 36874 and 36938 Da, which appear to be the same as those reported earlier (36879 and 36936 Da).<sup>27</sup> All the measured masses agree with retention of the two Zn<sup>+</sup> ions associated with the protein.

Concanavalin A, the jack bean lectin, crystallizes as a tetramer,<sup>29</sup> but shows a dimer-tetramer equilibrium in solution which is both temperature and pH dependent.<sup>30</sup> Early mass spectrometry experiments showed evidence of monomers, dimers and tetramers depending on spray conditions.<sup>31</sup> Our preparation of ConA in 10 mM NH<sub>4</sub>OAc (Fig. 3(c)) has a complicated spectrum with three distinct charge envelopes representing monomer, dimer and tetramer ions. Under our spray conditions, the major ions are from the monomer with charges 6+ to 10+. Less abundant are ions from the dimer (14+, 15+ and 16+) with the 15+ appearing between the 7+ and 8+ ions of the monomer. Well separated from the monomer-dimer envelope is the third ion envelope, containing 20+ to 23+ ions of a tetramer. Addition

of small amounts of urea to the buffer changes the relative charge on the ions, but not their relative abundances, so that now the most abundant monomer ion is the 7+ and that of the tetramer is the 20+.

## DISCUSSION

The experiments described here show that millimolar amounts of urea affect the charge envelopes of four different noncovalent protein complexes, and change the stoichiometry of some of the complexes. One explanation for this 'urea effect' is a competition for charge between the protein and the urea during the desolvation stage of ion formation, a mechanism similar to that proposed by Heck's group for the proton sponges.<sup>9</sup> Most of their proteins showed only a loss of charge in response to the particular additive. However, ions from the glutamate synthase dimer were observed only when ammonium acetate buffer was used, and, under very harsh conditions, myoglobin lost its heme.<sup>9</sup> Our spectra of ConA show the simplest effect of urea; a loss of charge and no change in the stoichiometry. For HPII and ADH we see the loss of transient conformations (the octamer and dimer, respectively), which we speculate are formed by weak interactions that are easily disrupted by the urea. It should be noted that both these proteins retain their cofactors – heme in HPII and Zn<sup>+</sup> in ADH – even in the presence of urea. The citrate synthase protein is a hexamer by crystallization<sup>32</sup> and SEC,<sup>12</sup> but the dimer is an important part of the allosteric process.<sup>13,21,32</sup> Only H-bonds and van der Waals forces hold the three dimers together,<sup>32</sup> and the presence of urea could interfere with those contacts so that the hexamer falls apart. At low concentrations of urea and 20 mM ammonium bicarbonate buffer, both the dimer and hexamer ions lose charge so that both ion envelopes shift to higher  $m/z$ . No monomer ions are observed, so it could be argued that these conformations have become more compact in response to the stabilizing effect of urea proposed by Bhuyan.<sup>11</sup> By reducing the concentration of buffer ions to 5 mM, we have produced a more fragile hexamer (because NH<sub>4</sub><sup>+</sup> acts as a mimic to K<sup>+</sup>, the normal activator), thus increasing the effect of the urea so that less urea is needed to reduce the charge on the dimers and decrease the intensity of hexamer ions. Additional urea results in the appearance of monomers, an indication of denaturation. In the absence of urea, monomers are only produced under extreme voltage conditions or with the addition of acid. None of the 'standard' biochemical assays of citrate synthase showed any effect of low concentration of urea, probably because they are done at higher concentration of protein which shifts the dimer-hexamer equilibrium to the hexamer. These experiments with urea show the sensitivity of mass spectrometry assays to minor perturbations of protein structure that cannot be determined by any other means. Urea is simple to use, unlike the 'proton sponges', and it gives meaningful information about noncovalent complexes, information that may aid in the understanding of protein structure and stability. Even the small amounts of urea compatible with electrospray and nanospray ionization are sufficient to weaken some subunit interactions and thus may be of assistance in determining the stoichiometries of natural and unnatural complexes.

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