All-or-none solvent-induced transitions between native, molten globule and unfolded states in globular proteins

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Background: It has long been established that temperature-induced melting of small globular proteins is an all-or-none transition. Little was known, however, about the degree of cooperativity of denaturant-induced transitions in proteins, especially in those cases in which the proteins unfold through the molten globule state.

Results: We have processed data on the equilibrium urea-induced and guanidinium chloride (GdmCl)-induced unfolding of globular proteins from the native to the unfolded state, from the native to the molten globule state and from the molten globule to the unfolded state. We show that in all these cases, the cooperativity of unfolding increases linearly with the increase of the molecular weight of the protein up to 25–30 kDa.

Conclusions: The cooperative unit of the urea-induced and GdmCl-induced equilibrium transitions of small proteins between the native, molten globule and unfolded states includes the protein molecule as a whole. In other words, both native and molten globule proteins are unfolded by strong denaturing solvents according to an all-or-none mechanism.

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Introduction

Cooperative melting (denaturation) of protein molecules protects their structure from destruction by thermal movement at native conditions and is, therefore, a necessary prerequisite of their enzymatic and some other functions. The extreme case of the cooperative transition is an all-or-none transition in which a cooperative unit includes the whole molecule, i.e. no intermediate states can be observed in the transition region. These transitions, which are analogous to the first-order phase transition in macroscopic systems, are of special physical and biological importance.

It has been shown that the denaturation of small globular proteins is an all-or-none transition [1], whereas large proteins consist of two or more domains that can melt independently of each other [2]. However, a full understanding of temperature melting of proteins is limited by uncertainty over the physical state of temperature-denatured proteins [3]. Unlike temperature-denatured proteins, proteins denatured by urea or guanidium chloride (GdmCl) are strongly unfolded, but much less is known about the cooperativity of solvent-induced denaturation. Tanford [3] and Privalov [1] have argued that solvent-induced denaturation is also an all-or-none process, and this assumption was later confirmed by size-exclusion chromatography for urea-induced denaturation of myoglobin [4] and bovine serum albumin [5] as well as for GdmCl-induced denaturation of staphylococcal nuclease [6], lysozyme and myoglobin [7,8].

A new situation arose when it was shown [9] that protein molecules can exist not only in the native and unfolded states, but also in the intermediate molten globule state [10–15]. Moreover, urea-induced or GdmCl-induced protein unfolding often involves at least two steps: the native to molten globule states (N⇄MG) and the molten globule to unfolded states (MG⇄U) transitions [10–12,15]. Both transitions are cooperative (as they follow an S-shaped curve), but the dimensions of cooperative units for these transitions were unknown and, as a consequence, it was unclear whether or not these transitions are all-or-none.

The usual method used to estimate the cooperativity of transition is to measure the slope of the transition curve at its middle point. This slope is proportional to the change of thermodynamic quantity conjugated with the variable provoking the transition: enthalpy difference for temperature-induced transitions, difference in the degrees of ionization for pH-induced transitions or difference in the numbers of ‘bound’ denaturant molecules for urea-induced or GdmCl-induced transitions. All of these parameters obtained from the breadth of transitions refer by definition to a cooperative unit, i.e. to a part of a molecule that undergoes the transition as a whole.

With the use of some techniques, it is also possible to measure the corresponding quantity for the whole protein molecule (or its monomer unit). A well known example is microcalorimetry, which allows the simultaneous measurement of transition enthalpies for the whole protein molecule and for the cooperative unit [1]. In an analogous way, the potentiometric titration allows the measurement of the difference in the degree of ionization for the whole molecule and comparison with the value for the cooperative unit [16]. Unlike these cases, it is practically impossible to measure the difference in the number of 'bound' denaturant molecules $\Delta\upsilon$ per protein molecule for urea-induced or GdmCl-induced transitions. To this end it would be necessary to measure not only the difference between urea or GdmCl activity of a dilute protein solution and of a solvent, but also to evaluate the change in this difference upon transition of the protein molecules.

We have used a different approach to study the character of the solvent-induced conformational transitions in proteins. This approach is to analyze how the slope of transition depends on the protein molecular weight (M). It is clear that the slope of phase transition in small systems depends on the dimensions of this system [17,18]. In the case of first-order phase transition, the slope increases proportionally to the number of units in a system [17], whereas the slope for second-order phase transition is proportional to the square root of this number [18]. This means that it is possible to distinguish between phase and non-phase intramolecular transitions by measuring whether their slopes depend on molecular weight. For phase transitions, moreover, it is even possible to determine whether it is of the first or of the second order. Of course, this approach can work only for small globular proteins, as the existence of multidomain structure in large proteins may lead to more or less independent unfolding of different domains.

In this paper (but see also a preliminary communication [19]), we analyze the existing experimental data on urea-induced and GdmCl-induced N$\rightleftarrows$U, N$\rightleftarrows$MG and MG$\rightleftarrows$U transitions in small proteins and conclude that all three types of transition are of all-or-none character. This implies that the molten globule state is a real third thermodynamic state of protein molecules separated by all-or-none transitions from two other states.

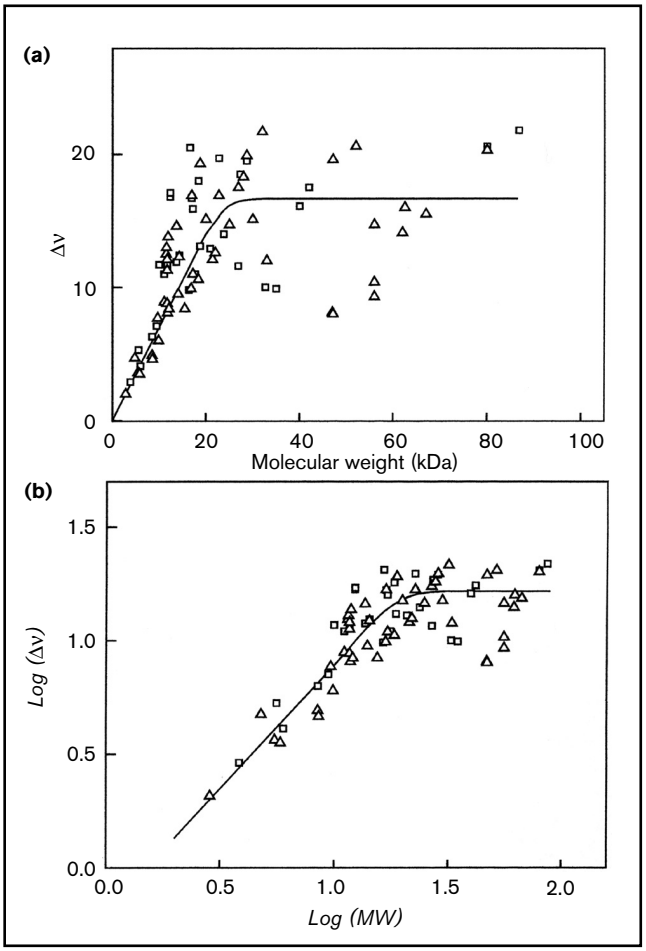
Results

Molecular weight dependence of the cooperativity of urea-induced and GdmCl-induced N$\rightleftarrows$U transitions in globular proteins: evidence that the unfolding of small proteins is an intramolecular analog of phase transition

Figure 1a presents the available data on the molecular weight dependence of the degree of cooperativity of urea-induced and GdmCl-induced N$\rightleftarrows$U transitions for 90 proteins with a wide range of molecular weights. The value $\Delta\nu^{eff}$ (see Materials and methods) was used as the measure of cooperativity. The curve $\Delta\nu^{eff}$ (M) comprises two parts. For small globular proteins (with M<25–30 kDa), cooperativity of transition increases with M, whereas for large proteins (with M>25–30 kDa), cooperativity does not depend on M. The use of a logarithmic scale makes such behavior even more obvious (see Fig. 1b).

The existence of clear molecular weight dependence for the degree of cooperativity shows that denaturant-induced unfolding of small globular proteins exhibits the characteristics of phase transition. On the other hand, the independence of the cooperativity of unfolding from M at M>25–30 kDa appears to be related to the multidomain structural organization of large proteins (see above).

Figure 1



Molecular weight dependence of cooperativity parameters for urea- and GdmCl-induced N$\rightleftarrows$U transitions in proteins. **(a)** $\Delta\nu^{eff}_{N\rightleftarrows U}$ versus M dependence, where M is protein molecular weight (in kDa) and $\Delta\nu^{eff}_{N\rightleftarrows U}$ is the difference between the numbers of denaturant molecules 'bound' to the unfolded and the native state of a cooperative unit (see equation 6). **(b)** Log($\Delta\nu^{eff}_{N\rightleftarrows U}$) versus log(M) dependence. Squares and triangles refer to urea- and GdmCl-induced transitions respectively.

It is interesting that $\Delta\nu^{eff}$ (M) dependencies for N⇄U transitions induced by urea and GdmCl coincide within experimental errors (see Fig. 1). The same behavior is also observed for N⇄MG and MG⇄U transitions (see below). This may mean that the number of binding sites or the area of exposed residue surface (but not the energy of the binding!) is the same for urea and GdmCl. Alternatively, both these denaturants may change the structure of a solvent in a similar manner.

All-or-none character of solvent-induced N⇄U, N⇄MG and MG⇄U transitions in small globular proteins

N⇄U transitions

As mentioned above, the dependence of the slope of transition curve on the number of units in a system is evidence of the phase character of this transition. Moreover, we can distinguish the intramolecular analog of phase transitions of the first and the second order by measuring the dependence of their slopes on molecular weight — transition is of the first order if its cooperativity is proportional to M [17] and of the second order if it is proportional to $M^{1/2}$ [18].

Figure 2 presents the $\log(\Delta\nu^{eff})$ versus log(M) dependence for solvent-induced N⇄U transitions in small globular proteins. Statistical analysis of this dependence shows that it can be described by equation 1:

$$\log(\Delta\nu^{eff}_{N\rightleftarrows U}) = 0.97 \log(M) - 0.07 \qquad (1)$$

with the root mean square deviation (rms)=0.112 and correlation coefficient r=0.87.

As the proportionality coefficient in the above equation is equal to 0.97±0.15, we can conclude that $\Delta\nu^{eff}$ for N⇄U transitions is proportional to M. In other words, the urea-induced and GdmCl-induced unfolding of small globular proteins is an all-or-none transition, i.e. an intramolecular analog of first-order phase transition in macroscopic systems.

N⇄MG transitions

A number of proteins unfold not via a two-state (N⇄U) transition but through the molten globule intermediate. In these cases, two transitions — from the native to the molten globule state (N⇄MG) and from the molten globule to the unfolded state (MG⇄U) — can be monitored by different experimental techniques (for example by near and far UV circular dichroism, respectively). Therefore, it is possible to study N⇄MG and MG⇄U transitions separately even in the cases in which they overlap.

Figure 3 presents the $\log(\nu^{eff})$ versus log(M) dependence for N⇄MG transitions in small globular proteins for which urea-induced or GdmCl-induced unfolding proceeds through the molten globule state. Despite a much smaller number of experimental points, the slope of the transition curve can also be seen to increase with the molecular weight of a protein. This increase can be approximated by equation 2:

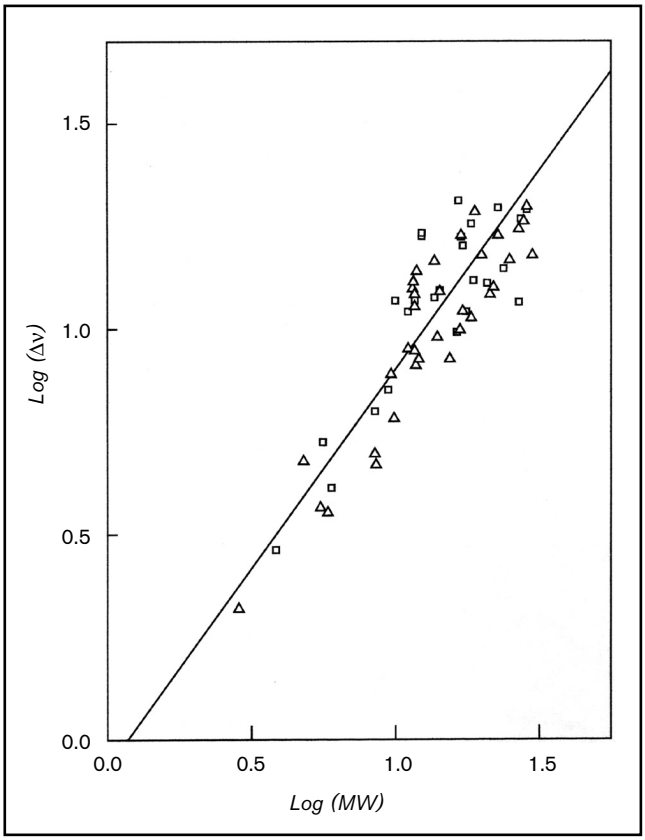
$$\log(\Delta\nu^{eff}_{N\rightleftarrows MG}) = 1.02 \log(M) - 0.49 \qquad (2)$$

with rms=0.090 and r=0.82. In this case, the proportionality coefficient between $\log(\nu^{eff})$ and log(M) is equal to 1.02±0.15. This means that the transition from the native to the molten globule state is also an all-or-none transition, i.e. the intramolecular analog of phase transition of the first order.

MG⇄U transitions

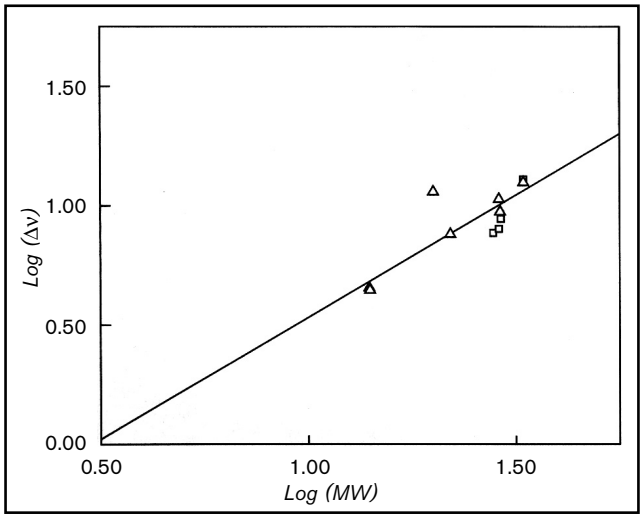
Figure 4 shows the $\log(\Delta\nu^{eff})$ versus log(M) dependence for proteins that undergo urea-induced or GdmCl-induced unfolding of the molten globule state (MG⇄U transi-

Figure 2



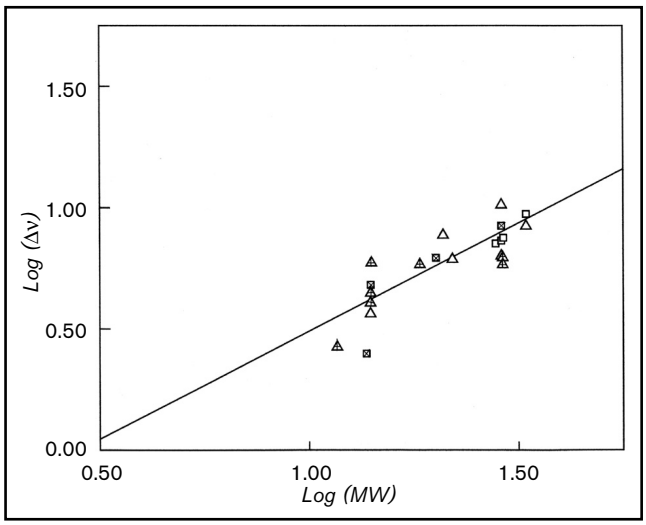
Molecular weight dependence of the difference between the numbers of denaturant molecules 'bound' to the completely unfolded and to the native states ($\Delta\nu^{eff}_{N\rightleftarrows U}$) for small globular proteins (M up to 25–30 kDa) presented in double logarithmic scale. Squares and triangles refer to urea-induced and GdmCl-induced transitions respectively.

Figure 3



Molecular weight dependence (up to 25–30 kDa) of the difference between the numbers of denaturant molecules, 'bound' to the molten globule and to the native states ($\Delta\nu^{eff}_{N\rightleftarrows U}$). Squares and triangles refer to urea-induced and GdmCl-induced transitions respectively.

Figure 4



Molecular weight dependence (for small globular proteins) of the difference between the numbers of denaturant molecules 'bound' to completely unfolded and to the molten globule states ($\Delta\nu^{eff}_{N\rightleftarrows U}$). Crossed symbols refer to the unfolding of acid-induced molten globules (see the text). Squares and triangles refer to urea-induced and GdmCl-induced transitions respectively.

tions). It shows that $\Delta\nu$ for MG⇄U transitions depends on molecular weight and that this dependence can be described by equation 3:

$$\log(\Delta\nu^{eff}_{MG\rightleftarrows U}) = 0.89\log(M) - 0.40 \quad (3)$$

with rms=0.092 and r=0.84. One can see that even for this kind of transition the coefficient of proportionality between $\log(\nu^{eff})$ and log(M) is close to unity (0.89±0.16). This means that the transition between two denatured states — the molten globule and the unfolded state — is an all-or-none transition, i.e. the intramolecular analog of phase transition of the first order.

This result clearly contradicts the belief that all denatured states of proteins are identical from the thermodynamic point of view [1] and that this is true even in the case of them being structurally different [20].

Figure 4 includes two types of MG⇄U transition. The first type refers to proteins for which urea-induced or GdmCl-induced unfolding goes through the molten globule state, and the second type refers to proteins that can be transformed into the molten globule state by low pH and then unfolded by urea or GdmCl. The absence of systematic differences between the $\Delta\nu^{eff}$ (M) dependencies for these two types of transition suggests that the thermodynamic properties of the urea-induced or GdmCl-induced and pH-induced molten globules are similar.

The all-or-none character of N⇄MG and MG⇄U transitions is confirmed also by the fact that the sum of $\Delta\nu^{eff}$ for these transitions is close to $\Delta\nu^{eff}$ for N⇄U transitions for proteins of similar molecular weight [19]. It is clear that this can be the case only if the cooperative unit for all these kinds of transition is the same.

Reliability of results

It would be possible for the proportionality between cooperativity of transition and molecular weight to be a result of the dependence of $\Delta\nu^{eff}$ on denaturant activity in the middle of transition (a_t) and/or the dependence of a_t value on molecular weight of proteins. However, the analysis of available data shows that this is not the case. Indeed, Figure 5a shows that $\Delta\nu^{eff}$ values for N⇄U transitions in small globular proteins, from 2.2 to 22 kDa, do not depend on urea or GdmCl activity in the middle of transition (a_t). Values for a_t are distributed between ~0.3 and ~8.0 without any systematical dependence on M (see Fig. 5b). This means that the cooperativity of transition does not depend on a_t and a_t does not depend on molecular weight.

Discussion

'All-or-none' character of urea-induced and GdmCl-induced N⇄U, N⇄MG and MG⇄U transitions in small globular proteins

The all-or-none character of denaturant-induced N⇄U transitions in globular proteins was predicted many years ago [1,3]. This suggestion was substantiated for a number of proteins by the coincidence of transition curves moni-

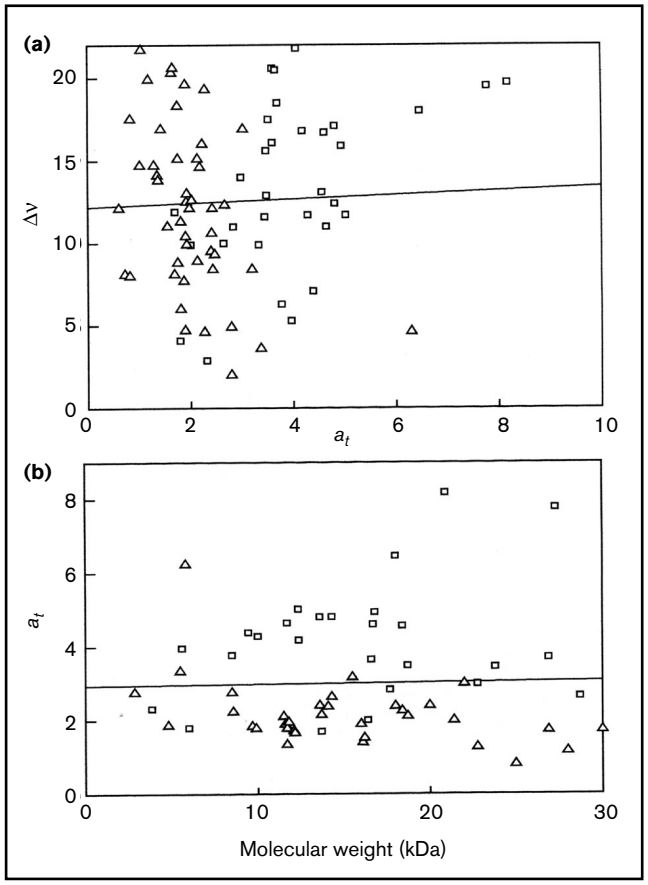
tored with independent structural probes. Later, it was directly proved for four proteins by the observation of bimodal distribution on molecular dimensions within the transition region [4–8]. However, the nature of N⇄MG and especially MG⇄U transitions was much more obscure. It had already been shown [21] that the temperature denaturation of human α-lactalbumin is an all-or-none process. As the melting of this protein is not accompanied by large changes in either compactness or secondary structure, α-lactalbumin at high temperature is in the molten globule state. Thus, N⇄MG transition in this protein is of an all-or-none character. It is important to emphasize that it is difficult to distinguish intramolecular analogs of the first and the second order phase transitions by the dependence of the average properties of a molecule on a denaturing agent. There are only two relatively simple possible ways of doing this. The first is to study the dependence of the slope of transition on molecular weight. The second is to use separation techniques, such as size-exclusion chromatography, which will show two peaks for slow all-or-none transition, whereas for an intramolecular analog of second-order phase transition it will show only one broad peak. Using this second approach, we have shown that GdmCl-induced unfolding of the molten globule at low temperatures for carbonic anhydrase and β-lactamase is an all-or-none process [22,23].

Despite the earlier evidence, this study is the first one in which the existence of 'all-or-none' N⇄MG and MG⇄U transitions has been established as a general phenomenon. This means that the molten globule state is thermodynamically quite different from both the native and unfolded states. In other words, the molten globule state is by no means anything like a 'squeezed coil' but instead appears to be the third general phase state of protein molecules, in addition to their native and unfolded states.

The physical reason for the all-or-none character of protein denaturation (i.e. N⇄U and N⇄MG transitions) has been argued to be due to the cooperative destruction of a rigid tertiary structure [24], namely to the drastic change in intramolecular movements in the protein core — from rotation librations of small amplitude to the jumping of aliphatic side groups from one rotational isomer into another [25]. The physical reason for the all-or-none character of the molten globule⇄unfolded state transition is as yet unknown.

During the past few years, evidence has been accumulating that the molten globule state may play an important role in many physiological processes (see [15,26] for review). If so, the all-or-none character of the molten globule unfolding may have a biological role protecting the molten globule state in a living cell from uncooperative destruction by thermal fluctuation.

Figure 5



(a) Parameters of cooperativity of N⇄U transitions, $\Delta\nu^{eff}_{N\rightleftarrows U}$ for different activities of urea and GdmCl in the middle of transition (a_t). **(b)** Values of a_t for N⇄U transition at different molecular weights of proteins.

Materials and methods

Determination of a cooperativity for urea-induced and GdmCl-induced transitions

The equilibrium constant for all-or-none transition between two states, K^{eff}, is determined by equation 4:

$$K^{eff} = \frac{\Theta}{1 - \Theta} \qquad (4)$$

where Θ is the fraction of molecules in one of these states.

The measure of cooperativity for denaturant-induced transition is the value $\Delta\nu^{eff}$ – the difference between the numbers of denaturant molecules which are 'bound' to a cooperative unit in two states [3]. $\Delta\nu^{eff}$ can be obtained [27] from the equilibrium constant, K^{eff} , by equation 5:

$$\Delta\nu^{eff} = \left(\frac{\delta \ln K^{eff}}{\delta \ln a}\right)_{a=a_t} \qquad (5)$$

where a is the activity of a denaturing agent and a_t its activity in the middle point of transition. It follows from equations (4) and (5) that:

$$\Delta\nu^{eff} = 4a_t\left(\frac{\delta \Theta}{\delta a}\right)_{a=a_t} \qquad (6)$$

and therefore the value of $\Delta\nu^{eff}$ can be determined from the dependence of any protein parameter on the activity of a denaturant.

The activities of urea and GdmCl as functions of their molar concentrations (m) were calculated by the empirical equations [28]:

$$a_{urea}=0.9815\ (m)-0.02978\ (m)^2+0.00308\ (m)^3 \quad (7)$$

and

$$a_{GdmCl}=0.6761(m)-0.1468(m)^2+0.02475(m)^3+0.00132(m)^4 \quad (8)$$

We have considered transitions described by sigmoidal curves monitored by some of the following parameters: for N⇄U transitions, near and far UV CD, viscosity, biochemical activity, fluorescence, ^{1}H-NMR spectra, chromatography, and absorbance; for N⇄MG transitions, near UV CD, ^{1}H-NMR and biochemical activity; for MG⇄U transitions, far UV CD, viscosity, fluorescence, ^{1}H NMR spectra, chromatography, and absorbance. (For more information, see Tables 1 and 2 in the Supplementary material published with this paper on the internet.)

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