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Reshaping the folding energy landscape by chloride salt: impact on molten-globule formation and aggregation behavior of carbonic anhydrase

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Abstract **During chemical denaturation different intermediate states are populated or suppressed due to the nature of the denaturant used. Chemical denaturation by guanidine–HCl (GuHCl) of human carbonic anhydrase II (HCA II) leads to a three-state unfolding process ($C_{m,NI} = 1.0$ and $C_{m,IU} = 1.9$ M GuHCl) with formation of an equilibrium molten-globule intermediate that is stable at moderate concentrations of the denaturant (1–2 M) with a maximum at 1.5 M GuHCl. On the contrary, urea denaturation gives rise to an apparent two-state unfolding transition ($C_m = 4.4$ M urea). However, 8-anilino-1-naphthalene sulfonate (ANS) binding and decreased refolding capacity revealed the presence of the molten globule in the middle of the unfolding transition zone, although to a lesser extent than in GuHCl. Cross-linking studies showed the formation of moderate oligomer sized (300 kDa) and large soluble aggregates (>1000 kDa). Inclusion of 1.5 M NaCl to the urea denaturant to mimic the ionic character of GuHCl leads to a three-state unfolding behavior ($C_{m,NI} = 3.0$ and $C_{m,IU} = 6.4$ M urea) with a significantly stabilized molten-globule intermediate by the chloride salt. Comparisons between NaCl and LiCl of the impact on the stability of the various states of HCA II in urea showed that the effects followed what could be expected from the Hofmeister series, where Li^+ is a chaotropic ion leading to decreased stability of the native state. Salt addition to the completely urea unfolded HCA II also led to an aggregation prone unfolded state, that has not been observed before for carbonic anhydrase. Refolding from this state only provided low recoveries of native enzyme.**

Keywords: Intermediate; Denaturant; Misfolding

1. Introduction

The mechanism by which a protein polypeptide chain spontaneously folds to its native state is still a major goal to solve for protein chemists. Current research has focused on the role of folding intermediates or ensembles in reaching the native conformation. In this respect, the molten-globule intermediate has been extensively studied both by equilibrium and kinetic methods [1].

Folding studies of human carbonic anhydrase II (HCA II) have revealed the existence of folding intermediates both at equilibrium and kinetic conditions [2,3].

HCA II unfolds in two well-separated transitions when incubated in increasing concentrations of guanidine–HCl (GuHCl). This leads to the formation of a stable enzymatically inactive folding intermediate at medium concentrations of the denaturant [4,5]. Characterization of this intermediate has shown that it is of molten-globule type. In addition, it has been demonstrated that this molten globule is prone to aggregation and impossible to reactivate upon dilution of the GuHCl denaturant. The formed aggregates from the molten globule are soluble oligomers with a diameter of approximately 13.5 nm. The aggregation site is very specific, and is located in the large β-sheet of the protein, within a limited region between the hydrophobic β-strands 4 and 7 [6]. During refolding of the completely denatured protein, a molten-globule intermediate transiently appears [7] and gives rise to a fraction of protein that aggregates, which lowers the yield of native enzyme to about 75% under optimal conditions [8–10]. However, the chaperonin GroEL binds to the molten-globule intermediate and protects it from aggregating during refolding, but cannot rescue preformed aggregates [6,11].

In this report, we have studied how a non-ionic denaturant as urea unfolds HCA II and what role chloride salts have on this process. Surprisingly, urea denaturation leads to an apparent two-state unfolding transition, although closer examination revealed minor formation of the molten-globule intermediate also in this case. Moreover, it is shown that chloride ions markedly stabilizes this molten globule that thereby promotes self-assembly into various oligomeric states.

2. Materials and methods

2.1. Chemicals

Urea and LiCl were purchased from Merck and the urea concentration was determined by refractive index [12]. 8-Anilino-1-naphthalene sulfonic acid (ANS) was obtained from Sigma and GuHCl from Pierce. All other chemicals were of reagent grade.

2.2. Protein production and purification

The cysteine-free C206S pseudo wild-type type of HCA II was expressed from BL21/DE3 *Escherichia coli*. Protein production and purification were performed as described by Freskgård et al. [13]. We

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Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; GuHCl, guanidine–HCl; HCA II, human carbonic anhydrase II

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have previously shown that the pseudo wild-type has properties that are indistinguishable from those of the wild-type of HCA II [4].

2.3. Stability measurements

The unfolding profile of HCA II was determined by intrinsic Trp fluorescence measurements as previously described [14]. Before measurements, the enzyme was incubated for 24 h at 23 °C in freshly made urea solutions (up to 8 M) with and without NaCl or LiCl (1.5 M) buffered with 10 mM Na-phosphate, pH 7.5.

The stability data were analyzed by a non-linear least-square fitting equation through linear extrapolation to 0 M of denaturant as described by Santoro and Bolen [15], using the program TableCurve 2D (Jandel Scientific). The midpoint concentration of denaturation, C_m, of each unfolding transition and the free energy of unfolding in water ΔG_{H_2O} were determined as previously reported [16].

2.4. ANS binding

The binding of ANS to protein in its molten-globule state [17,18] was examined by incubation of protein (0.025 mg/ml; 0.83 μM) at various concentrations of urea with and without NaCl or LiCl (1.5 M) in 10 mM Na-phosphate buffer, pH 7.5, with an 80-fold molar excess of ANS present for 24 h at 23 °C. Fluorescence emission spectra were recorded between 450 and 650 nm and the intensity at emission maximum of the samples was plotted as a function of urea concentration. The samples were excited at 360 nm, and excitation and emission bandwidths were 5 and 10 nm, respectively. The cuvette length was 1 cm and the spectrofluorimeter was thermostated at 23 °C.

2.5. Chemical cross-linking

The used glutaraldehyde cross-linking protocol was used essentially as that described for transthyretin [19].

Samples of HCA II (0.20 mg/ml) were incubated for about 24 h in different concentrations of urea, in the presence of 0, and 1.5 M NaCl (or in 1.5 M GuHCl) in 10 mM Na-phosphate buffer, pH 7.5. Glutaraldehyde (25%, 100 μl) was added to 1.0 ml of the HCA II solution and the cross-linking reaction was allowed to proceed for 4 min before the reaction was quenched by addition of 100 μl of $NaBH_4$ (7% in 0.1 M NaOH). The cross-linked protein samples were desalted using a PD10 column (Amersham Biosciences) in water and were thereafter mixed with reducing SDS cocktail (final SDS concentration was 2.5%). The samples were boiled for 5 min before loading onto a SDS–PAGE gel. The protein bands were stained with Coomassie, scanned and quantified by use of the program Scion Image (Scion Corp.).

3. Results and discussion

3.1. Less formation of molten globule by urea than by GuHCl denaturation

The stability of HCA II towards urea denaturation, as monitored by the change in intrinsic Trp fluorescence, is shown in Fig. 1A. Contrary to GuHCl-induced unfolding the protein seems to be unfolded in a two-state process without presence of an intermediate state with a mid-point concentration of unfolding (C_m) at 4.4 M urea. Notably, urea unfolding of bovine carbonic anhydrase II (BCA II) did not lead to any detectable equilibrium intermediates, whereas denaturation by GuHCl gave rise to several unfolding intermediates [20]. The magnitude of the fluorescence red-shift indicates that HCA II is denatured to the globally unfolded state [21]. The calculated free energy (ΔG_{H_2O}) of the $N \leftrightarrow U$ transition is 19.0 kcal/mol, which is very similar to the summed free energies (18.7 kcal/mol) of the GuHCl-mediated transitions ($N \leftrightarrow I \leftrightarrow U$) [4]. This also supports the notion that the protein is unfolded to the same degree by the two denaturants.

In the transition zone some binding of the hydrophobic probe ANS is noted, indicating that minor formation of the molten-globule intermediate also occurs during urea denaturation (Fig. 1B). In comparison BCA II did not show any ANS

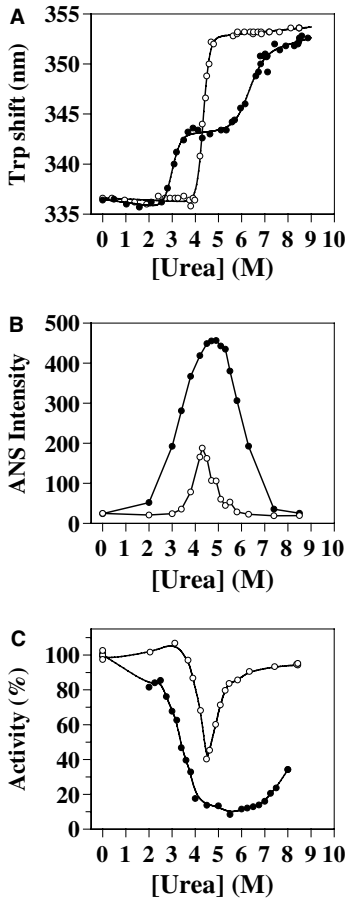

Fig. 1. Stability and molten-globule formation in urea and urea + 1.5 M NaCl. (A) Global unfolding measured by Trp fluorescence emission peak of HCA II (0.025 mg/ml) following excitation at 295 nm. Open circles show data from HCA II incubated in urea and filled circles show data from incubation in urea + 1.5 M NaCl. The data were fitted to a single or double transition for HCA II unfolded in urea or in urea + 1.5 M NaCl, respectively. (B) ANS binding to probe for the formation of molten globules. ANS was excited at 360 nm and the emission intensity at 480 nm was recorded. The intensity was corrected from reference samples in the corresponding concentrations of urea or urea + 1.5 M NaCl, respectively. Open circles show data from HCA II incubated in urea and filled circles show data from incubation in urea + 1.5 M NaCl. (C) Refolding yield of HCA II after incubation in various concentrations of urea. Reactivation of the enzyme was initiated through dilution of the denaturant to 1.0 M urea and 0.025 mg/ml HCA II following 24 h incubation at the urea concentrations indicated on the *x*-axis (at 0.25 mg/ml). Incubation was performed in urea alone (open circles) or in urea + 1.5 M NaCl (filled circles).

binding in the range 0–8 M urea [20]. Previously, we have observed that the molten-globule intermediate of HCA II is prone to aggregation at equilibrium conditions. Thus, if HCA II is denatured at GuHCl concentrations promoting the molten-globule state (1–2 M) the recovery of active enzyme upon dilution of the denaturant is drastically reduced. This is due to irreversibly formed self-assembled aggregates by the molten-globule intermediates [6]. Denaturation by urea concentrations (4–5 M) leading to some ANS binding also gives rise to significantly decreased refolding yields (~40%) upon renaturation under optimal conditions (i.e., dilution to 1 M urea [22] (Fig. 1C), although not to such low yields as for GuHCl (~10%) [6]). Together the ANS and refolding experiments

clearly show that a molten-globule intermediate of HCA II also is induced by moderate concentrations of urea, but to a lesser extent than for GuHCl. In addition, cross-linking experiments show that this molten globule, formed at 4.5 M urea, is highly prone to aggregate, with a major fraction giving a strong 300 kDa SDS–PAGE band corresponding to approximately decameric sized aggregates. Moreover, a considerable fraction forms very large aggregates (>1000 kDa), seen as a protein band that has not been able to leave the concentration gel (Fig. 2, lane 3).

Under optimal denaturation and renaturation conditions, i.e., at 8 and 1 M urea, respectively, a reactivation yield of about 90% can be achieved. This is somewhat higher than can be obtained after GuHCl renaturation (about 75%) [11], indicating that the kinetic molten-globule, that seems to cause aggregation, also is suppressed in urea. However, complete reactivation is not obtained, probably due to formation of a minor fraction of aggregates of relatively small size evident from "smeared" bands (60–300 kDa) in the cross-linking analysis of HCA II in 7 M urea (Fig. 2, lane 5).

3.2. Salt effects on the conformational states of HCA II during urea unfolding

Since urea is a non-ionic denaturant and GuHCl is ionic containing Cl^- as counter ions, it is of interest to investigate the addition of Cl^- containing salts to urea to understand the difference in unfolding behavior of HCA II by the two denaturants. Since 1.5 M GuHCl leads to maximal formation of the aggregation-prone molten-globule form according to ANS and reactivation data [6], we included 1.5 M Cl^- in the urea solutions to mimic the ionic character of GuHCl either by addition of NaCl or LiCl.

3.3. NaCl addition stabilizes the molten globule

Addition of 1.5 M NaCl to varying concentrations of urea transforms the equilibrium unfolding of HCA II to a clear three-state unfolding process ($N \leftrightarrow I \leftrightarrow U$), as monitored by Trp fluorescence measurements (Fig. 1A) with a profile that is very similar to that induced by GuHCl [6]. ANS binding to this intermediate is, similar to the GuHCl-induced intermediate [23], profound and is dramatically increased by the addition of NaCl to the urea denaturant (Fig. 1B). Furthermore, this intermediate is almost totally impossible to reactivate upon dilution to refolding conditions (Fig. 1C). Thus, it exhibits all the characteristics that are typical for the molten-globule state of HCA II. Cross-linking performed on the protein (in 4.5 M urea + 1.5 M NaCl) demonstrates that the molten globule forms aggregates to a larger extent than in the absence of salt (Fig. 2, lane 4) leaving only 20% of the protein in monomeric form. Some differences are obvious regarding the distribution of aggregate sizes. Thus, much less oligomeric 300 kDa aggregates and more of the very large aggregate type are formed than in urea without added salt. In 1.5 M GuHCl no monomers remain, but >95% decameric self-assembled oligomers (300 kDa) are formed with <5% in large aggregates, indicating that GuHCl makes the molten globule self-assembly more monodisperse (Fig. 2, lane 2).

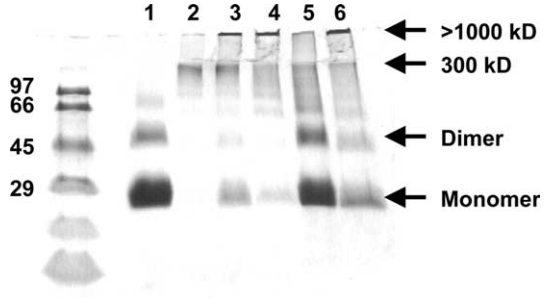

Fig. 2. SDS–PAGE gel of cross-linked protein. HCA II (0.2 mg/ml) was incubated (24 h, 23 °C) in various denaturants buffered with 10 mM phosphate buffer (pH 7.5) prior to 4 min of glutaraldehyde cross-linking. Lane 1: Native protein no denaturant; Lane 2: 1.5 M GuHCl; Lane 3: 4.5 M urea; Lane 4: 4.5 M urea + 1.5 M NaCl; Lane 5: 7 M urea; Lane 6: 7 M urea + 1.5 M NaCl.

The presence of NaCl stabilizes the aggregation prone molten globule at the expense of the native (N) and the unfolded (U) states, since the $N \leftrightarrow I$ transition is shifted from 4.4 to 3.0 M urea and the $I \leftrightarrow U$ transitions from 4.4 to 6.4 M urea. For acid-unfolded cytochrome *c* and apomyoglobin anions have been shown to stabilize molten globules by reduction of the net positive charge on the protein through anion binding [24]. Anion shielding by chloride of local repulsive positive charges can dramatically stabilize quaternary structures which was demonstrated for transthyretin [19]. At pH 7.5 HCA II has almost no net charge, since the p*I* is 7.3 [25]. However, the HCA II molecule contains several tertiary ionic interactions. Therefore, specific anion binding could have significant effects in breaking tertiary interactions in the native conformation, and thereby destabilize the native state. On the other hand, Debye shielding of fluctuating positively charged side chains in the molten-globule intermediate can have a stabilizing effect in that it reduces charge repulsion between these groups. Moreover, increased ionic strength will strengthen the hydrophobic effect, which also will favor the molten-globule state over the unfolded state. Previously, we have demonstrated that the molten-globule forms aggregates in a very specific way mediated by hydrophobic interaction [6].

Presence of NaCl at high concentrations of urea (>7 M) appears to induce a globally unfolded protein (U) according to Trp emission maximum (353 nm) [21] and low ANS binding (Fig. 1A and B), nevertheless only modest degrees of reactivation (15–30%) could be achieved from these U-states (Fig. 1C). The lowered yield of reactivateable enzyme is also in this case due to aggregation of the unfolded protein chain. The cross-linking experiment performed on protein incubated in 7 M urea with 1.5 M NaCl shows that a high proportion of the protein forms aggregates of varying sizes with predominance of very large aggregates (Fig. 2, lane 6). This is the first time that carbonic anhydrase has been observed to aggregate in the unfolded state, which seems to be promoted by an increased hydrophobic effect from NaCl. This clustering could originate from the hydrophobic residues in the central core of HCA II that evidently can be solubilized by 5 M GuHCl but not by 7 M urea containing 1.5 M NaCl.

3.4. Effects of LiCl

The effects on urea denaturation of HCA II by addition of LiCl might shed some light on the mechanism by which Cl^- and the counter ion influence the stability of the various states of HCA II. According to the Hofmeister series, Li^+ is a more salting-in cation than Na^+ and consequently expected to be more denaturing. As seen in Fig. 3A, the native state is also even more destabilized in the presence of LiCl than with NaCl

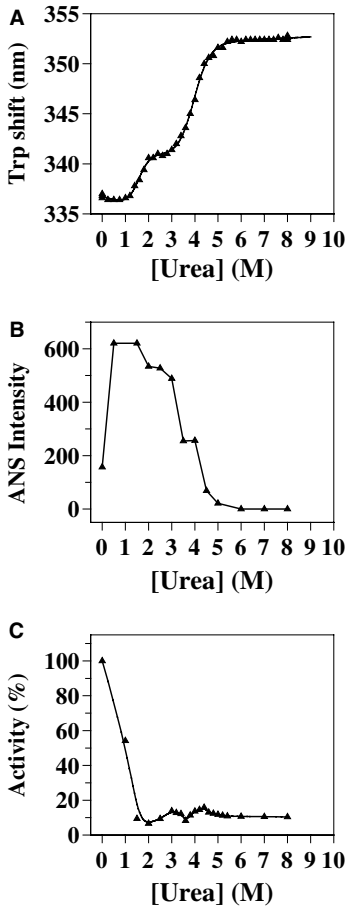

Fig. 3. Stability and molten-globule formation in urea + 1.5 M LiCl. (A) Global unfolding measured by Trp fluorescence emission peak of HCA II (0.025 mg/ml) following excitation at 295 nm. The data were fitted to a double transition. (B) ANS binding to probe for formation of molten globules. ANS was excited at 360 nm and the emission intensity at 480 nm was recorded. The intensity was corrected from reference samples in the corresponding concentrations of urea + 1.5 M LiCl. (C) Refolding yield of HCA II after incubation in various concentrations of urea + 1.5 LiCl. Reactivation of the enzyme was initiated through dilution of the denaturant to 1.0 M urea and to 0.025 mg/ml HCA II following 22 h incubation at the urea concentrations indicated on the *x* axis (at 0.25 mg/ml).

($C_{m,N\leftrightarrow I}$ = 1.6 M urea). The second unfolding transition occurs at 3.9 M urea, thus Li^+ increases the denaturing effect of urea compared to Na^+ by improving solvation of exposed hydrophobic and peptide groups in the unfolded state as predicted from the lyotropic or Hofmeister series [26–30]. Furthermore, the total stability of HCA II in 1.5 M LiCl, calculated for the two unfolding transitions ($N \leftrightarrow I \leftrightarrow U$) and extrapolated to 0 M urea, is only 10.5 kcal/mol compared to 19.0 kcal/mol in the absence of LiCl, i.e., in water. The corresponding stability in 1.5 M NaCl is 21.2 kcal/mol. This clearly indicates that Li^+ has a destabilizing effect on the protein, whereas Na^+ does not significantly affect the global stability. However, it should be pointed out that the figures above do not represent true equilibrium values, since irreversible aggregation also occurs. Nevertheless, these values give at least qualitative information supporting the conclusions drawn above.

The unfolding intermediate, found at 2–3 M urea with 1.5 M LiCl, binds ANS (Fig. 3B) and is practically impossible to reactivate (Fig. 3C), which means that in the presence of LiCl also an aggregation prone molten globule is formed. Notably, ANS binding even occurs in the $N \leftrightarrow I$ pre-transition zone, indicating that the native conformation becomes more flexible exposing otherwise buried hydrophobic patches due to the decreased hydrophobic interaction caused by LiCl. This is also evident from the reactivation measurements. At high urea concentrations with LiCl no reactivation of globally unfolded protein (λ_{max} = 352 nm for Trp fluorescence) can be obtained (Fig. 3C), indicating that LiCl like NaCl in urea leads to the formation of stable aggregates. In the reactivation and cross-linking experiments, the protein was incubated at higher concentrations (0.25 and 0.20 mg/ml, respectively) than in the corresponding Trp fluorescence and ANS experiments (0.025 mg/ml) and a visible precipitate of the protein was in fact observed from samples at high urea concentrations (7 M), showing that the protein is undergoing aggregation. Accordingly, cross-linking experiments were spoiled due to precipitation of very large aggregates. Together it is evident that addition of LiCl to urea makes the U state of HCA II even more prone to aggregate than NaCl does. This was highly surprising since LiCl was expected to resemble GuHCl more than NaCl.

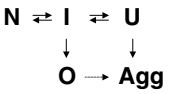

Fig. 4. Cartoon showing the folding and misfolding pathways for HCA II that is greatly influenced by the denaturant used. N, native protein; I, molten-globule intermediate; U, unfolded protein; O, oligomeric (majority is decameric) self-assembled molten globules; Agg, large soluble aggregates >1000 kDa formed from packed unfolded chains and molten globules.

3.5. Conclusions

The scheme in Fig. 4 summarizes the folding and misfolding pathways for HCA II. The ionic character of the denaturants markedly affects the stability of the molten-globule intermediate in HCA II and influences the self-assembly properties of the partially unfolded protein. Promoting the formation of the aggregation-prone molten-globule intermediate by the presence of chloride salts favors self-assembly into misfolded oligomeric structures that cannot be refolded. Furthermore, the ionic effect strengthens the hydrophobic interaction resulting in increased association in the urea-unfolded state into large aggregates.

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