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# Counteracting Effects of Renal Solutes on Amyloid Fibril Formation by Immunoglobulin Light Chains*

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**In primary (light chain-associated) amyloidosis, immunoglobulin light chains deposit as amyloid fibrils in vital organs, especially the kidney. Because the kidney contains high concentrations of urea that can destabilize light chains as well as solutes such as betaine and sorbitol that serve as protein stabilizers, we investigated the effects of these solutes on *in vitro* amyloid fibril formation and thermodynamic stability of light chains. Two recombinant light chain proteins, one amyloidogenic and the other nonamyloidogenic, were used as models. For both light chains, urea enhanced fibril formation by reducing the nucleation lag time and diminished protein thermodynamic stability. Conversely, betaine or sorbitol increased thermodynamic stability of the proteins and partially inhibited fibril formation. These solutes also counteracted urea-induced reduction in protein thermodynamic stability and accelerated fibril formation. Betaine was more effective than sorbitol. A model is presented to explain how the thermodynamic effects of the solutes on protein state equilibria can alter nucleation lag time and, hence, fibril formation kinetics. Our results provide evidence that renal solutes control thermodynamic and kinetic stability of light chains and thus may modulate amyloid fibril formation in the kidney.**

Primary systemic antibody light chain (AL)[1] amyloidosis is a plasma cell disorder in which immunoglobulin light chains deposit pathologically as amyloid fibrils in the body, leading to progressive organ failure and eventual death (1–3). Amyloid fibrils consist of the variable domain, $V_L$, or the $V_L$ and a contiguous portion of the constant domain of λ or κ light chains (1–4). Light chains that are amyloidogenic *in vivo* are less thermodynamically stable than those that are nonamyloidogenic (5–8). *In vitro* there is a strong inverse relationship between the intrinsic thermodynamic stability of light chains and their propensity to form fibrils (2, 5–8). Furthermore, thermodynamic stability and propensity to form fibrils have been modulated extrinsically by employing solutes such as urea and sucrose (6, 8, 9).

It has been hypothesized that a partially unfolded conformation, the aggregation-competent species, fosters nucleation and subsequent fibrillization of proteins (5, 6, 8, 10–12). Certain mutations can dispose benign proteins to amyloid fibril formation by enhancing formation of this partially unfolded state (5–8, 11, 13–15). However, mutations are not always necessary for amyloidosis (*e.g.* with β2-microglobulin in dialysis patients and with transthyretin during senile systemic amyloidosis) (11, 16, 17), presumably because in certain cases the wild-type protein population contains aggregation-competent species. In addition, properties of the aqueous environment such as pH, temperature, ionic strength, and the presence of chaotropic agents may also be important parameters affecting levels of partially unfolded species and, hence, in amyloidosis (5, 6, 11, 13, 18). *In vivo*, partially denaturing environments can be found, for example, in the lysosome (acidic pH) and in the kidney, which has high intra- and extracellular concentrations of urea (11, 19–21). Lysosomal extracts can convert amyloidogenic immunoglobulins into amyloid fibrils *in vitro* (22). *In vitro* exposure of transthyretin to acidic pH, mimicking that of the lysosome, causes partial unfolding, which in turn promotes fibril formation (17). The conversion of $V_L$s into amyloid fibrils under acidic conditions proceeds through at least one partially unfolded intermediate (5). Thus, both intrinsic stability of proteins and effects of local solution environments are important criteria for the determination of whether light chains will form amyloid fibrils.

The kidney is the major target organ for light chain deposition, which can be found in various forms: fibrillar (AL-amyloidosis), punctate (light chain deposition diseases), crystalline (acquired Fanconi's syndrome), and amorphous (tubular cast neuropathy) (1, 3, 23). For AL-amyloidosis, about one-third of patients have amyloid fibrils in the kidney (24). Amyloid is found in all compartments of the kidney, with the glomerulus being a primary site of fibril deposition (1, 23). In about 10% of the cases, amyloid is restricted to nonglomerular regions, especially the inner renal medulla (25). Urea is found at high concentrations (0.4 M-1.5 M) in the inner renal medulla, especially during antidiuresis (26). A common feature in AL-amyloidosis patients is Bence Jones proteinuria, in which high levels of monoclonal κ or λ light chains are excreted in the urine, although urinary concentrations of light chains do not necessarily correlate with pathogenesis (24). As a component of

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[1] The abbreviations used are: AL-amyloidosis, light chain-associated amyloidosis; $V_L$, immunoglobulin light chain variable domain; PBS, phosphate-buffered saline; ThT, thioflavine T; GdnHCl, guanidine hydrochloride; HPLC, high performance liquid chromatography.

the glomerular filtrate, light chains are exposed to various concentrations of urea during processing by the kidney. Exposure to urea is expected to enhance fibril formation by thermodynamically favoring the population of aggregation-competent, partially unfolded conformations of light chains (6–9). As a result of this effect, for example, a light chain variant with an intrinsic thermodynamic stability that is just sufficient to avoid amyloid fibril deposition in other areas of the body, might form fibrils in the renal medulla.

However, the kidney also contains high concentrations of stabilizing osmolytes that can counteract destabilization by urea, such as betaine, glycerophosphocholine, sorbitol, and inositol (19–21, 27, 28). Counteraction of urea-induced protein structural and functional perturbations by osmolytes has only been demonstrated with a few enzymes (19–21, 27, 28). We hypothesize that this phenomenon will also occur with immunoglobulin light chains, because the mechanisms for the effects of urea and stabilizing osmolytes are nonspecific (28). Urea destabilizes the compact, native state of proteins because it binds preferentially to the protein backbone, and protein species with greater solvent exposure are thermodynamically favored (28, 29). Stabilizing osmolytes are preferentially excluded from the surface of proteins, which shifts the unfolding equilibrium to favor compact states (28, 30–33). Osmolytes (*e.g.* glycerol and sucrose), in the absence of urea, have been found to inhibit amyloid fibril formation from scrapie prion protein (34) and immunoglobulin light chains (8) by stabilizing the native states and reducing the levels of the aggregation-competent species.

To examine the possible roles of renal solutes in AL amyloidosis and to gain further insight into the relationships between the thermodynamic stability of light chains and their propensity to form amyloid fibrils, we modulated protein stability by varying concentrations of renal solutes, *i.e.* urea, betaine, and sorbitol. The proteins used, recombinant $V_L$ SMA and $V_L$ LEN (6, 10), represent prototype amyloidogenic and nonamyloidogenic proteins, based on previous *in vivo* and *in vitro* observations (6, 10). It has previously been established that recombinant SMA forms fibrils *in vitro* that are indistinguishable from *in vivo* light chain fibrils, and recombinant LEN does not form fibrils *in vitro* in buffer alone (6, 10).

## EXPERIMENTAL PROCEDURES

*Materials*—Betaine, sorbitol, and guanidine hydrochloride were purchased from Sigma, and ultrapure urea was purchased from ICN. Before use, concentrated urea solutions were treated with a chelating resin (C-7901, Sigma) for 1 h and then with a mixed-bed ion exchange resin (AG501-X8, Bio-Rad) for 1 h to remove ions (21, 27, 29). The solution was then filtered using 0.2-$\mu$m nylon filters (Fisher) and finally freeze-dried in an FTS Durastop lyophilizer (Stone Ridge, NY). The urea concentration was determined by measuring the refractive index of the solution (35). Other chemicals were purchased from Sigma and were of reagent or higher grade.

*Proteins Expression and Purification*—The recombinant $V_L$ protein SMA has the same sequence as the $V_L$ that originated from lymph node-derived amyloid fibrils of a patient (SMA) who had AL amyloidosis (10). The recombinant $V_L$ protein LEN has the same sequence as a $V_L$ isolated from the urine of a patient (LEN) with multiple myeloma who, despite excretion of up to 50 g of this protein daily, had no renal dysfunction or evidence of amyloidosis (9, 10). Both proteins were products of same germline gene family $\kappa$ IV (10). The $V_L$ sequence of SMA differs from that of LEN by only eight amino acid residues (6, 10). Recombinant $V_L$s SMA and LEN were expressed in *Escherichia coli* and purified by the methods of Wilkins-Stevens *et al*. (10) and Raffen *et al*. (6) with the following modifications. Instead of 5-ml prepacked Econo-Pac Q and S cartridges, High Q and S resins (Bio-Rad) were packed in 1 × 50-cm glass columns (Bio-Rad). Fractions were eluted with 8× volume (400 ml), 0–900 mM NaCl gradient for SMA and 8× volume (400 ml), 0–150 mM NaCl gradient for LEN. Fractions were collected and assayed by SDS-polyacrylamide gel electrophoresis. The fractions containing $V_L$s were pooled and stored at 4 °C in PBS buffer (10 mM potassium phosphate, pH 7.4, with 100 mM NaCl). The purity of the $V_L$s exceeded 99% (based on SDS-polyacrylamide gel electrophoresis). Protein concentration was determined using extinction coefficients of 1.71 and 1.82 (mg $ml^{-1}$ $cm^{-1}$) at 280 nm for SMA and LEN, respectively, which were calculated from the amino acid sequence (6).

*In Vitro Fibril Formation and Sample Analyses*—Fibrils were produced *in vitro* with incubation at 37 °C and agitation at 250 rpm as described by Kim *et al*. (8). 1 mg/ml protein was incubated in PBS buffer (pH 7.4, with 10 mM potassium phosphate plus 100 mM NaCl), PBS plus various urea concentrations (0.5, 1, and 1.5 M), PBS plus 0.5 M betaine or sorbitol (for SMA), and PBS plus urea (0.5 and 1 M) containing either 0.5 M betaine or sorbitol. All buffer solutions contained 0.1% sodium azide to inhibit microbial growth. Samples were analyzed at designated time points for soluble protein using size exclusion high performance liquid chromatography (HPLC) and for fibrils, using a Thioflavin T (ThT) assay (36), as described by Kim *et al*. (8). Size exclusion HPLC analysis documented that before incubations, the proteins (1 mg/ml) were dimers, free of higher molecular weight light chain aggregates. Fibril formation kinetics were analyzed by fitting time-dependent changes in ThT fluorescence of incubated samples to the following nonlinear, least squares sigmoidal equation.

$$F_{ThT} = A/(1 + \exp[-B(t\text{-}t_i)]) \quad \text{(Eq. 1)}$$

where $F_{ThT}$ is the fluorescence intensity of ThT, $A$ is the ThT fluorescence intensity in the post-transition plateau, $t_i$ is the inflection point, *i.e.* the midpoint of the transition region, $B$ ($h^{-1}$) is the fibril growth rate constant, and $t$ is the time in h. The lag time ($t_{lag}$) of fibrillogenesis was calculated by extrapolation of the linear region of the sigmoidal transition phase of ThT fluorescence assay to the abscissa intercept (7).

*Guanidine Hydrochloride (GdnHCl) Unfolding*—The free energy of denaturation, $\Delta G$, was measured using tryptophan fluorescence at 25 °C after samples were equilibrated with various concentrations of GdnHCl overnight at room temperature (8). Protein concentration was 10 $\mu$g/ml. GdnHCl concentrations were determined using the refractometer (35). The $C_m$ value, the midpoint of unfolding transition region, was calculated by complex sigmoid nonlinear analysis (8). The m and $\Delta G$ values were calculated as the slope and ordinate intercept, respectively, of a linear regression of $\Delta G$ *versus* GdnHCl concentration (35, 37).

*Intrinsic Fluorescence*—Intrinsic fluorescence emission spectra were measured with an Aviv model ATF105 spectrofluorometer at a sample temperature of 25 °C. The samples were excited at 295 nm, and the emission was monitored from 300 to 400 nm. Excitation and emission slit widths were set at 5 and 10 nm, respectively. Protein solutions (10 $\mu$g/ml) were prepared in PBS and in 0.5, 1.0, and 1.5 M urea in PBS and equilibrated overnight at room temperature before measurement of fluorescence emission spectra. Two scans of each solution were averaged, and the appropriate spectrum of the buffer solution was subtracted from this average.

*Infrared (IR) Spectroscopy and Transmission Electron Microscopy of Fibrils*— IR spectra were measured with a Bomem MB-series spectrometer (Bomem) purged with dry air from a Balston dryer (Balston) to remove water vapor. Soluble proteins (20 mg/ml) and fibrils (~5 mg/ml) were measured in a BioTools liquid sampling cell equipped with $CaF_2$ windows that provided a 6-$\mu$m path length. Before measurement, fibrils formed in various solution conditions were separated from unaggregated protein and washed by two rounds of centrifugation and resuspension with PBS buffer. Spectra were analyzed according to the previously established criteria (38, 39). Transmission electron microscopy on fibrils was performed by the method of Raffen *et al*. (6).

## RESULTS

*Fibril Formation in PBS*—Fig. 1 shows time courses for soluble native protein concentrations and ThT fluorescence, which is indicative of fibril formation (6, 7, 36). In PBS buffer alone, the amount of soluble SMA started to decrease after 3 days of incubation, and soluble protein was undetectable 1 day later (Fig. 1*A*). During this period, there was a concomitant increase in ThT fluorescence, indicative of fibril formation (Fig. 1*B*). Conversely, LEN remained soluble and did not form fibrils (Fig. 1, *C* and *D*) in PBS buffer until 15 days (data not shown) of incubation. The fibril formation profile for SMA showed a characteristic nucleation-dependent polymerization pattern, having an initial lag phase followed by a rapid growth phase (7, 13, 40, 41). SMA in PBS buffer had a lag time of 66 h and a

fibril formation rate constant of 0.32 $h^{-1}$.

*Effects of Urea on Fibril Formation*—Before assessing effects of urea on fibril formation, we determined whether physiological concentrations of urea (*i.e.* up to 1.5 M) are sufficient to cause structural perturbation of SMA and LEN. Unfolding curves were constructed by measuring intrinsic fluorescence intensity (8) as a function of urea concentrations (data not shown). The onset of unfolding was at 2.9 and 4.1 M urea for SMA and LEN, respectively. The midpoint of the unfolding transition was at 4.1 and 5.2 M urea, respectively, for SMA and LEN. Thus, even at the highest urea concentration tested (1.5 M) in the fibril formation experiments, both proteins were native. Furthermore, fluorescence emission spectra for SMA and LEN in 0.5, 1.0, and 1.5 M urea were unperturbed relative to the respective spectrum for each protein in PBS (data not shown). For both proteins, neither the wavelength of the fluorescence emission maximum nor the emission spectrum width at half maximum intensity was affected by urea.

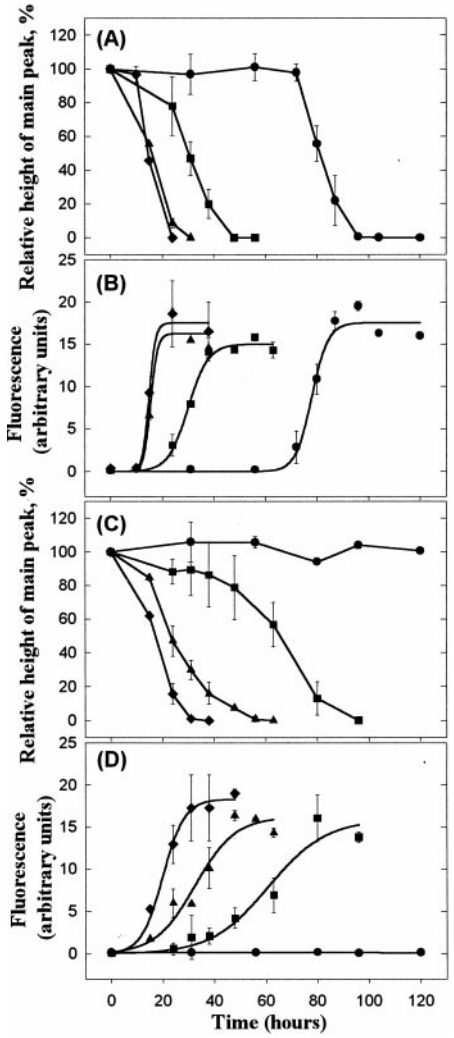


FIG. 1. **Effects of urea on soluble protein levels measured by size exclusion HPLC and fibril formation measured by ThT fluorescence for SMA (*A* and *B*) and LEN (*C* and *D*) during incubation at 37 °C with agitation.** The symbols represent the following buffer conditions: ●, PBS buffer; ■, 0.5 M urea; ▲, 1 M urea; ♦, 1.5 M urea. In *B* and *D*, the *solid lines* were drawn by nonlinear, least square fits of the data using Equation 1. Data points represent the mean ± S.D. for triplicate incubated samples.

When SMA and LEN were incubated in physiological concentrations of urea (0.5, 1, and 1.5 M), both proteins formed fibrils (Fig. 1). Urea dramatically decreased the lag time compared with that in PBS buffer alone (Table I), with the highest urea level resulting in the shortest lag times. At 0.5 and 1 M urea concentration, SMA displayed much shorter lag phases than LEN. In 1.5 M urea, both proteins had similar lag times (Table I).

*Effects of Stabilizing Osmolytes on Fibril Formation*—To test the hypothesis that stabilizing osmolytes can counteract the effects of urea on fibril formation, incubations were conducted in betaine or sorbitol and mixtures of urea and betaine or sorbitol. In the absence of urea, 0.5 M betaine or sorbitol partially inhibited fibril formation by SMA compared with that in PBS alone (Fig. 2). The lag phase was much longer, and the fibril growth rates were slower than those for the protein in PBS alone (Table I). Betaine and sorbitol were not tested on LEN in PBS alone because the protein did not form fibrils over the 5-day time course in just PBS. When both proteins were incubated in mixtures of urea and betaine or sorbitol, the stabilizing osmolytes partially counteracted the acceleration of fibril formation induced by urea. The lag times were lengthened relative to values measured in urea alone. However, there were not significant differences in fibril growth rates between the mixtures of urea and betaine or sorbitol and urea alone (Table I). Mixtures with 1:1 molar ratios of osmolytes to urea were more effective at counteracting urea-mediated fibril formation than those with 1:2 molar ratios. Betaine was much more effective at inhibiting fibril nucleation than sorbitol, either in the presence or absence of urea.

*Characterization of Fibrils*—Fibrils formed under all of the various buffer conditions showed the typical characteristics of

TABLE I
*Calculated kinetic parameters for the fibril formation of SMA and LEN*

The calculated values represent the mean ± S.E. for triplicate samples.

| Buffers | SMA | | LEN | |
|---|---|---|---|---|
| | $t_{lag}$[a] | Growth rate[b] | $t_{lag}$[a] | Growth rate[b] |
| | *h* | $h^{-1}$ | *h* | $h^{-1}$ |
| PBS | 65.7 ± 1.4 | 0.32 ± 0.05 | NC[c] | NC[c] |
| 0.5 M sorbitol | 87.1 ± 0.6 | 0.14 ± 0.02 | | |
| 0.5 M betaine | 112.3 ± 0.9 | 0.11 ± 0.02 | | |
| 0.5 M urea | 20.3 ± 0.8 | 0.26 ± 0.03 | 38.6 ± 2.4 | 0.09 ± 0.02 |
| 1 M urea | 9.4 ± 0.9 | 0.75 ± 0.68 | 21.5 ± 0.9 | 0.13 ± 0.02 |
| 1.5 M urea | 8.8 ± 1.0 | 0.80 ± 0.75 | 8.8 ± 1.0 | 0.34 ± 0.05 |
| 0.5 M urea + 0.5 M sorbitol | 21.2 ± 0.4 | 0.23 ± 0.02 | 50.6 ± 1.2 | 0.16 ± 0.02 |
| 0.5 M urea + 0.5 M betaine | 34.3 ± 0.3 | 0.27 ± 0.04 | 58.1 ± 0.6 | 0.15 ± 0.02 |
| 1 M urea + 0.5 M sorbitol | 14.1 ± 0.5 | 0.36 ± 0.06 | 26.8 ± 0.9 | 0.20 ± 0.02 |
| 1 M urea + 0.5 M betaine | 14.7 ± 0.2 | 0.58 ± 0.21 | 39.0 ± 0.4 | 0.52 ± 0.13 |

[a] The lag time was calculated by the extrapolating of linear region of the sigmoidal transition region of ThT fluorescence assay to the abscissa (7).
[b] The fibril growth rate was determined by the B parameter obtained from the non-linear least square analysis, using the Equation 1, for the ThT fluorescence assay.
[c] Not calculated.

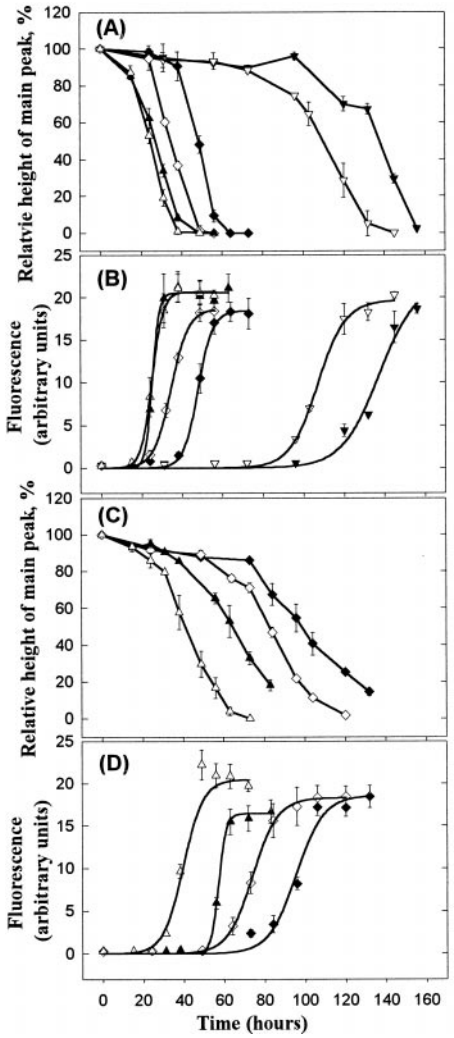


FIG. 2. **Effects of betaine or sorbitol with or without urea on soluble protein levels and fibril formation for SMA (*A* and *B*) and LEN (*C* and *D*) during incubation at 37 °C with agitation.** The symbols represent the following buffer conditions: ▼, 0.5 M betaine; (▽),0.5 M sorbitol; ♦, 0.5 M urea plus 0.5 M betaine; ◇, 0.5 M urea plus 0.5 M sorbitol; ▲, 1 M urea plus 0.5 M betaine; △, 1 M urea plus 0.5 M sorbitol. In *B* and *D*, the *solid lines* were drawn by nonlinear, least square fits of the data using Equation 1. Data points represent the mean ± S.D. for triplicate incubated samples.

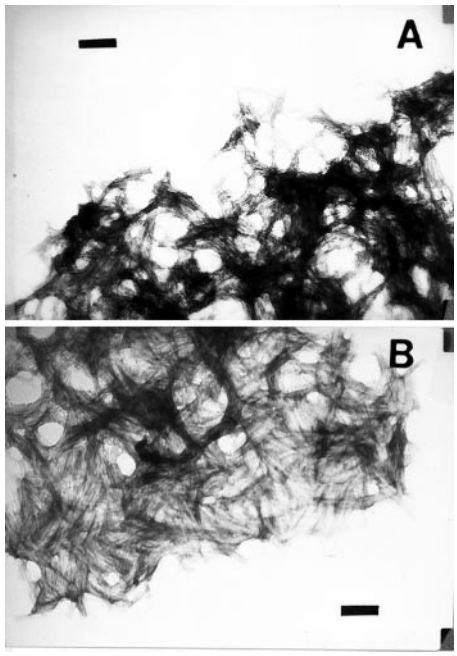


FIG. 3. **Transmission electron microscopy of negatively stained SMA fibrils produced in PBS buffer (*A*) and in 1 M urea buffer (*B*).** *Scale bars* represent 100 nm.

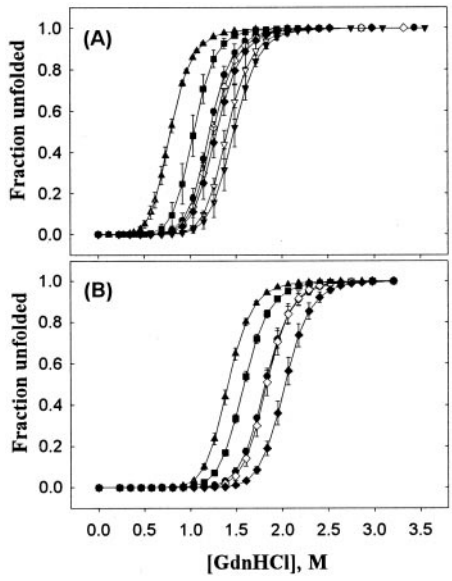


FIG. 4. **Guanidine-HCl unfolding curves in various solutes conditions for SMA (*A*) and LEN (*B*).** The symbols represents the following buffer conditions: ●, PBS buffer; ■, 0.5 M urea; ▼, 0.5 M betaine; ▽, 0.5 M sorbitol; ♦, 0.5 M urea plus 0.5 M betaine; ◇, 0.5 urea plus 0.5 M sorbitol; ▲, 1 M urea. Data points represent the mean ± S.D. for triplicate samples.

amyloid fibrils, based on transmission electron microscopy (Fig. 3) and IR spectroscopy (data not shown). Fig. 3 shows representative electron photomicrographs of SMA fibrils formed in PBS alone and in 1 M urea. Both sets of fibrils consist of linear, unbranched structures. Based on transmission electron microscopy, the fibrils formed from each of the two proteins, under all of the various buffer conditions, were morphologically indistinguishable (data not shown). IR spectra of fibrils of SMA and LEN showed characteristic intermolecular $\beta$-sheet bands, *i.e.* peaks around 1625 and 1695 $cm^{-1}$ (8, 39, 42), in the amide I region (data not shown).

*Effect of Urea and Stabilizing Osmolytes on the Free Energy of Denaturation*—Prompted by previous studies (2, 5–8) that demonstrated a strong inverse relationship between thermodynamic stability of light chains and fibril formation propensity, free energies of denaturation for SMA and LEN were measured under the same solution conditions used for the fibril formation studies. GdnHCl-induced unfolding curves are shown in Fig. 4, and Table II lists the calculated denaturation midpoints ($C_m$), differences in $C_m$ relative to values in PBS alone ($\Delta C_m$), free energies of denaturation ($\Delta G$), and differences in free energy of denaturation relative to that in PBS alone ($\Delta\Delta G$). In PBS alone, $C_m$ and $\Delta G$ values, respectively, were 1.2 M GdnHCl and 5.5 kcal/mol for SMA and 1.8 M GdnHCl and 7.2 kcal/mol for LEN (Table II), confirming previous studies showing that SMA is thermodynamically less stable than LEN (6). Urea decreased the thermodynamic stability of both proteins as measured by $C_m$ and $\Delta G$ (Fig. 4 and Table II). Betaine and sorbitol increased the values of $C_m$ and $\Delta G$ for SMA and counteracted the effects of urea on both proteins (Fig. 4 and Table II). Betaine increased $C_m$ and $\Delta G$ more than sorbitol. In mixtures containing 1:1 molar ratios of urea to betaine or sorbitol, the effects of the solutes on $C_m$ and $\Delta G$ values for SMA were approximately additive (Table II). For SMA, $C_m$ and $\Delta G$ in the 1:1 molar

TABLE II

*Thermodynamic parameters of SMA and LEN in various solute conditions, measured by GdnHCl unfolding experiments*

The calculated values represent the mean ± S.E. for triplicate samples.

| Proteins | Buffers | $C_m$[a] | $\Delta C_m$[b] | −m[c] | ΔG[c] | ΔΔG[b] |
|---|---|---|---|---|---|---|
| | | *M* | *M* | *kcal/mol/M* | *kcal/mol* | *kcal/mol* |
| SMA | PBS buffer | 1.21 ± 0.02 | | 4.51 ± 0.20 | 5.53 ± 0.25 | |
| | 0.5 M urea | 1.05 ± 0.01 | −0.16 ± 0.02 | 4.87 ± 0.18 | 5.14 ± 0.21 | −0.39 ± 0.33 |
| | 1 M urea | 0.81 ± 0.02 | −0.40 ± 0.03 | 4.64 ± 0.16 | 3.70 ± 0.15 | −1.83 ± 0.29 |
| | 0.5 M sorbitol | 1.43 ± 0.02 | 0.22 ± 0.03 | 4.28 ± 0.18 | 6.08 ± 0.24 | 0.55 ± 0.35 |
| | 0.5 M betaine | 1.50 ± 0.02 | 0.29 ± 0.03 | 4.16 ± 0.17 | 6.21 ± 0.24 | 0.68 ± 0.35 |
| | 0.5 M urea + 0.5 M sorbitol | 1.26 ± 0.02 | 0.05 ± 0.03 | 4.42 ± 0.23 | 5.59 ± 0.25 | 0.06 ± 0.35 |
| | 0.5 M urea + 0.5 M betaine | 1.31 ± 0.03 | 0.10 ± 0.04 | 4.52 ± 0.22 | 5.88 ± 0.23 | 0.35 ± 0.34 |
| LEN | PBS buffer | 1.82 ± 0.02 | | 3.97 ± 0.14 | 7.23 ± 0.22 | |
| | 0.5 M urea | 1.59 ± 0.01 | −0.23 ± 0.01 | 4.04 ± 0.13 | 6.44 ± 0.19 | −0.79 ± 0.29 |
| | 1 M urea | 1.43 ± 0.02 | −0.39 ± 0.02 | 4.18 ± 0.09 | 5.97 ± 0.18 | −1.26 ± 0.28 |
| | 0.5 M urea + 0.5 M sorbitol | 1.84 ± 0.02 | 0.02 ± 0.02 | 4.24 ± 0.11 | 7.79 ± 0.22 | 0.55 ± 0.31 |
| | 0.5 M urea + 0.5 M betaine | 2.06 ± 0.02 | 0.24 ± 0.02 | 3.98 ± 0.18 | 8.11 ± 0.20 | 0.87 ± 0.30 |

[a] $C_m$ was calculated by complex sigmoid, nonlinear regression analysis (8).
[b] $\Delta C_m$ and $\Delta\Delta G$ values were the differences in $C_m$ and $\Delta G$ values relative to those in PBS, respectively.
[c] The m and $\Delta G$ values were calculated as the slope and ordinate intercept, respectively, of a linear regression of $\Delta G$ *versus* GdnHCl concentration (35, 37).

mixtures were higher than those in PBS alone because the stabilizing effects of 0.5 M betaine or sorbitol were greater than the destabilization caused by 0.5 M urea.

## DISCUSSION

Our results document that urea decreases the thermodynamic stability of both SMA and LEN and decreases the lag time for nucleation of fibril formation. Betaine and sorbitol counteract the thermodynamic destabilization caused by urea, explaining why these solutes are able to offset, at least in part, the enhanced fibril formation induced by urea. To understand the mechanism(s) for these effects one must consider the different nonspecific interactions of urea *versus* betaine or sorbitol with proteins (*cf.* Ref. 28).

Urea is a destabilizer of native protein structure and generally an inhibitor of enzyme function (19, 29, 35). Destabilization of the native conformation is due to preferential binding of urea to protein backbone and other polar groups (28, 29). Urea binding decreases protein chemical potential and is directly proportional to solvent-exposed protein surface area. Thus, urea shifts the equilibrium away from the native state, favoring partially or fully unfolded states with greater solvent exposure than the native state. Urea also favors dissociation of higher order assemblies (*e.g.* native oligomers or fibril nuclei), because the total solvent-exposed surface area of the constituent monomers is greater than that for the assembled state (28).

Conversely, both betaine and sorbitol have been shown to increase the stability of several proteins against stresses such as high temperature (19, 21, 30) due to preferential exclusion from the protein surface (28, 30, 31, 33). Preferential exclusion of solutes increases the protein chemical potential in direct proportion to the protein surface area. The magnitudes of preferential exclusion and increase in chemical potential are greater for the denatured state and partially folded species than for the native state. Thus, the free energy barrier between the states increases, thereby stabilizing the native state (28, 31–33). Concomitantly, preferentially excluded solutes also thermodynamically favor higher order protein assemblies, which have a reduced surface area relative to the total for the constituent monomers (28).

Preferential interactions of urea and osmolytes with protein surfaces are nonspecific. Therefore, in mixtures of urea and osmolytes, the two thermodynamic effects should be compensatory and approximately additive (19, 21, 27–29). We found that the thermodynamic parameters, $\Delta C_m$ and $\Delta\Delta G$, in mixtures of urea and betaine or sorbitol were, within error of the determinations, equal to the sum of the individual values in each solute (Table I).

Betaine is a more effective stabilizer than sorbitol, either in the absence or presence of urea, both in terms of thermodynamic stability and its effect on the lag time for fibril nucleation (Tables I and II). In addition to the chemical nature of the protein surface, the chemical structure and physical properties of a solute determine its interactions (attractive or repulsive) with the protein and itself (28). The magnitude of protein chemical potential change caused by solute addition is determined by two parameters according to the following equation.

$$(\delta\mu_2/\delta m_3) = -(\delta m_3/\delta m_2)_{\mu_1\mu_3}(\delta\mu_3/\delta m_3)_{m_2} \quad \text{(Eq. 2)}$$

where $\mu_i$ and $m_i$ are the chemical potential and molal concentration of component $i$ (1 = water, 2 = protein, and 3 = solute), respectively (28). $\delta m_3/\delta m_2$ is the preferential interaction parameter, and its negative value indicates preferential exclusion (28). For bovine serum albumin, betaine has been found to have higher preferential exclusion ($\delta m_3/\delta m_2 = -39.6$ mol of betaine per mol of bovine serum albumin) than sorbitol ($\delta m_3/\delta m_2 = -9.8$ mol of sorbitol per mol of bovine serum albumin) (30, 31). The solute self-interaction parameter, $\delta\mu_3/\delta m_3$, is the other major component that determines the effect of solute on protein chemical potential (28). Calculation of self-interaction parameter using the activity coefficient of each solute (28, 33) gives a greater value for betaine ($\delta\mu_3/\delta m_3 = 776$ cal/mol$^2$) than for sorbitol ($\delta\mu_3/\delta m_3 = 591$ cal/mol$^2$). From Equation 2, the total effect of the two parameters shows that $\delta\mu_2/\delta m_3$ is 30.7 kcal/mol for betaine and 5.8 kcal/mol for sorbitol. Thus, betaine provides a greater increase in the protein chemical potential and, hence, in free energy of unfolding than sorbitol, and it will be more effective in counteracting destabilization by urea.

Amyloid fibril formation, including that for light chains (see "Results" and Ref. 7) is nucleation-dependent (13, 40, 41). To explain our results for the effects of urea and stabilizing osmolytes on light chain fibril formation, we propose a model that takes into account the effects of solutes on thermodynamic and kinetic aspects of the fibril formation pathway. Schematically, the native state, N, is in equilibrium with an aggregation-competent species, A, that is a partially unfolded form of N (5, 6, 8, 10–12, 43).

$$N \rightleftarrows A$$

SCHEME 1a

Furthermore, $n$ aggregation-competent species, $n$A, assemble to form a fibril nucleus, $A_n$ according to the following equation.

$$nA \rightleftarrows A_n$$

SCHEME 1b

Finally, fibrils, F, grow by the addition of protein molecules to the nucleus according to the following equation.

$$A_n + (N/A) \rightarrow F$$

SCHEME 1c

It is not known which species are added to the growing fibrils, N, A, or some other form of the protein.

The aggregation-competent species, A, may be a structurally perturbed monomer formed by dissociation of the native dimer or a dimer with altered tertiary structure. Because urea both favors protein dissociation and accelerates the rate of fibril formation and the stabilizing solutes betaine and sorbitol have the opposite effects, it seems plausible that light chain monomers could be the aggregation-competent species. However, if propensity to form fibrils was governed solely by the levels of monomers in solution, then LEN should form fibrils more readily than SMA, because the respective dimerization constants for the proteins are $4 \times 10^5$ M$^{-1}$ and $7 \times 10^5$ M$^{-1}$ (10). Even though at a given protein concentration LEN has a higher level of monomers than SMA, LEN is much more resistant to fibril formation than SMA (6, 10, Fig. 1). Furthermore, the light chain REC forms fibrils readily and has a dimerization constant of $2 \times 10^7$ M$^{-1}$ (6, 10). Thus, in comparisons between light chain variants, the levels of monomers do not correlate with propensities to form fibrils. Speculatively, within the population of monomers of SMA and REC there may be many more structurally perturbed molecules, which are prone to form nonnative aggregates leading to fibrils, than within the population of LEN monomers. If this were the case, alteration by urea and stabilizing osmolytes of the total SMA monomer level and the fraction of the monomer population that is aggregation competent could play an important role in governing the rate of fibril formation. Similarly, urea-induced perturbations of the LEN monomer structure and urea-induced increase in the total level of monomers might lead to a sufficient level of aggregation-competent monomers to promote aggregation and fibril formation. Based on this putative mechanism the critical factor governing propensity to form fibrils is the level of partially unfolded, aggregation-competent monomers and not the total level of monomers. The conformational perturbation needed to form aggregation-competent light chain monomers from native monomers is not known. Investigating this structural transition is an important area for future research.

Even though the structure of the aggregation-competent species, A, has not been elucidated, for our explanation about the effects of solutes on fibril formation, the main property that is assumed is that A has a greater surface area than N (on a per-monomer basis) and that higher temperatures (*e.g.* 37 *versus* 4 °C) also favor the more highly solvated expanded state, A.

The kinetics of formation of higher order species such as $A_n$ can be highly dependent on the concentration of A, especially if $n$ is a relatively large number. Thus, at low concentrations of A, the nucleation rate may be negligible, whereas above an apparent threshold concentration, $A_C$, the rate of nucleation increases dramatically (Fig. 5*A*). Thus, for our Scheme 1 the lag time for fibril formation is dependent on the time it takes to populate A to the threshold concentration, $A_C$ (Fig. 5*B*).

As depicted schematically in Fig. 5*A*, urea increases $A_C$ because protein assembly is less favorable in urea than in buffer alone (8, 28). Conversely, betaine or sorbitol favors assembly and, thus, a lower concentration of A is needed to foster nucleation; there is a decrease in $A_C$. If solutes were only affecting $A_C$, then one would predict that urea should slow fibril formation and betaine or sorbitol should speed the process, the opposite of our results (Figs. 1 and 2). In an earlier study on fibril formation from A$\beta$-peptide, it was found that stabilizing solutes such as trimethylamine-*N*-oxide and glycerol accelerated fibril formation (44). This could be because the solutes reduced $A_C$ and the overall process was not rate-limited by a step leading to the accumulation of A.

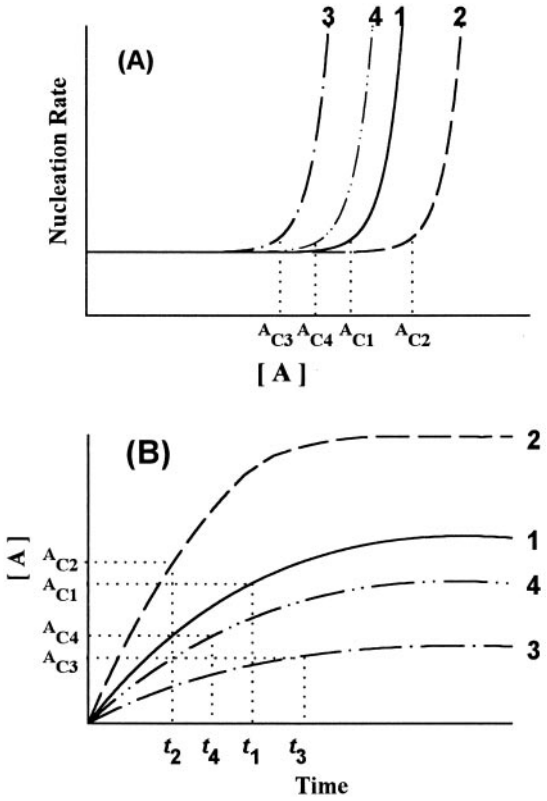


FIG. 5. **Schematic diagram showing the effect of various solutes on the threshold concentration of the amyloid-competent species, [A] for nucleation (*A*) and on the lag time to reach the threshold concentration, $A_C$ (*B*).** The *lines* shown are hypothetical and are presented as qualitative representations of the proposed effects of solute. $A_{C1}$, $A_{C2}$, $A_{C3}$, and $A_{C4}$ and $t_1$, $t_2$, $t_3$, and $t_4$ represent, respectively, the threshold concentrations of A for nucleation and the lag time required to reach those concentrations for each solute condition. Number subscripts for $A_C$ and $t$ represent the solution conditions of employed: *1*, *solid line*, buffer control in PBS; 2, *dashed line*, in the presence of urea; *3*, *dashed* and *single dotted line*, in the presence of a stabilizer (betaine or sorbitol); *4*, *dashed and double-dotted line*, in the presence of a 1:1 molar mixture of urea and betaine or sorbitol. See "Discussion" for a more detailed description.

However, according to our proposed pathway, the solutes can also affect the rate at which the aggregation-competent state, A, accumulates in solution (Fig. 5*B*). First, consider the protein in PBS alone. Upon transferring the sample from 4 to 37 °C, there is an increase in the level of A, as the system approaches the new equilibrium between N and A. The levels of A increase until the threshold concentration is reached and fibril formation is nucleated; the system may never actually reach equilibrium levels of A. The time from the initiation of the exposure to 37 °C to the point at which the threshold concentration is reached is equal to the lag time for fibril nucleation.

In the presence of urea, the equilibrium between N and A will be shifted toward A because of the greater solvent-exposed surface area of this species, and the level of A will increase more rapidly in urea than in PBS alone (Fig. 5*B*). As a result, even though the threshold concentration for nucleation, $A_C$, is higher in urea than in PBS, the time it takes to reach this concentration of A is less in urea. Thus, to explain our observed

reduction in duration of lag time in the presence of urea, the acceleration by urea of accumulation of A must predominate over the urea-induced increase in $A_C$. Note this is probably not the case in extremely high urea concentrations (*e.g.* 8 M) where $A_C$ is so high that fibril formation cannot be nucleated.

Conversely, betaine or sorbitol thermodynamically favors N over A and slows the rate of increase in level of A. Even though $A_C$ is lower in these solutes than in PBS, it takes a longer time to achieve this level of A, and the nucleation of fibrils is slowed. In this case the effect of the solutes on the rate of accumulation of A predominates over the solute-induced decrease in $A_C$.

This model also helps explain why betaine and sorbitol, at equimolar concentrations to urea, only partially offset the reduced lag time for fibril formation in urea, yet the thermodynamic stabilities of the SMA and LEN proteins were greater under these solution conditions than in PBS alone. Because the free energy of unfolding of the proteins in equimolar betaine or sorbitol plus urea was greater than that in buffer alone, the native state, N, is more favored in the mixed solute system than the denatured state or the A state. Accordingly, the threshold concentration of A and the rate of formation of A are reduced compared with that in PBS (Fig. 5). However, in contrast to the situation in betaine or sorbitol alone, in the mixed solute system the reduction in threshold concentration predominates, and the lag phase is shorter than that in PBS alone.

The arguments presented above can also be used to explain the effects of solutes of fibril formation by other pathways. For example, it may be that A first has to convert via an essentially irreversible step into a prenucleus species, P, which is in equilibrium with the nucleus, $P_n$. For immunoglobulin light chains this species could be a non-native dimer formed from structurally perturbed monomers (43, 45). For the purposes of our arguments, the key issue is that shifting the equilibrium between N and A toward A will increase the rate at which P appears. Conversely, employing solution conditions that thermodynamically favor N will slow the appearance of P. Alternatively, nucleation may depend on the time-dependent accumulation of covalently modified (*e.g.* with oxidized methionine residues) protein molecules. If formation of these modified species is also fostered by a protein conformational expansion, then counteracting solutes could modulate the rate of their generation and, hence, the rate of nucleation.

Finally, it is important to address the observations that $V_L$ LEN did not form amyloid fibrils *in vivo*, but it did so in the presence of urea *in vitro*. In addition to the effects of high concentrations of urea, agitation also accelerates *in vitro* fibril formation of both LEN and SMA. For example, in a 1 mg/ml solution of SMA, without agitation there were no fibrils detected, even after 17 days of incubation at 37 °C (data not shown). Furthermore, in the absence of agitation, LEN did not form fibrils even after 14 days of incubation at 37 °C in the presence of 1 M urea (data not shown). Solomon *et al.* (4) developed an *in vitro* fibrillogenesis assay that uses agitation to accelerate fibril formation of light chains and found a direct correlation between *in vivo* and *in vitro* fibril formation by light chains (4, 6–8). Agitation fosters protein aggregation mainly due to adsorption of the proteins at the air-liquid interface (46, 47). Adsorption may lead to both an increase in the local protein concentration and an alteration in protein structure at the interface, which can facilitate protein aggregation, including fibril formation. This physical stress is an important driving force to induce fibril formation of the proteins in *in vitro* systems, which is most likely not present *in vivo*.

In addition to properties of the protein itself and local environmental conditions, the effect of host factors, *e.g.* renal physiology, on fibril formation *in vivo* must be considered (9, 18, 23). Other constituents of amyloid fibrils such as proteoglycans and apolipoprotein-E may also be relevant (3, 9, 26). Thus, different behaviors could be seen with the same protein, depending on individual-specific factors.

Intrinsic thermodynamic stability, conferred by primary sequence and tertiary structure, and extrinsic stability, modulated by solution conditions such as pH, temperature, and stabilizing and destabilizing solutes, are all important factors for regulating fibril formation *in vivo* and *in vitro*. Kinetic factors, which are impacted by shifts in equilibria between protein species, are important as well because accumulation of a sufficiently high level of an aggregation-competent species is the critical step for nucleation and fibril formation. Physiological concentrations of urea strongly accelerate fibril formation of both pathologic and nonpathologic light chains predominantly by decreasing the time it takes to accumulate a nucleation-favoring concentration of an aggregation-competent species, implying that high concentrations of urea in the renal medulla may be at least one reason why the kidney is the most common site for AL-amyloidosis and other forms of pathological light chain deposition.

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REFERENCES

1. Kyle, R. A. (1994) *Annu. Rev. Med.* **45,** 71–77
2. Weltzel, R. (1997) *Adv. Protein Chem.* **50,** 183–242
3. Dhodapker, M. V., Melini, G., and Solomon, A. (1997) *Hematol. Oncol. Clin. North. Am.* **11,** 89–110
4. Solomon, A., Weiss, D. T., Murphy, C. L., Hrncic, R., Wall, J. S., and Schell, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9547–9551
5. Hurle, H. R., Helms, L. R., Li, L., Chan, W., and Wetzel, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5446–5450
6. Raffen, R., Dieckman, L. J., Szpunar, M., Wunschl, C., Pokkuluri, P. R., Dave, P., Wilkins-Stevens, P., Cai, X., Schiffer, M., and Stevens, F. J. (1999) *Protein Sci.* **8,** 509–517
7. Wall, J., Schell, M., Murphy, C., Hrncic, R., Stevens, F. J., and Solomon, A. (1999) *Biochemistry* **38,** 14101–14108
8. Kim, Y. S., Wall, J. S., Meyer, J., Murphy, C., Randolph, T. W., Manning, M. C., Solomon, A., and Carpenter, J. F. (2000) *J. Biol. Chem.* **275,** 1570–1574
9. Myatt, E. A., Westholm, F. A., Weiss, D. T., Solomon, A., Schiffer, M., and Stevens, F. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3034–3038
10. Wilkins-Stevens, P., Raffen, R., Hanson, D. K., Deng, Y. L., Berrios-Hammond M., Westholm, F. A., Murphy, C., Eulitz, M., Wetzel, R., Solomon, A., Schiffer, M., and Stevens, F. J. (1995) *Protein Sci.* **4,** 421–432
11. Kelly, J. W. (1998) *Curr. Opin. Struct. Biol.* **8,** 101–106
12. Ionescu-Zanetti, C., Khurana, R., Gillespie, J. R., Petrick, J. S., Trabachino, L. C., Minert, L. J., Carter, S. A., and Fink, A. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13175–13179
13. Wood, S. J., Wypych, J., Steavenson, S., Louis, J. C., Citron, M., and Biere, A. L. (1999) *J. Biol. Chem.* **274,** 19509–19512
14. Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C. F., and Pepys, M. B. (1997) *Nature* **385,** 787–793
15. McCutchen, S. L., Lai, Z., Miroy, G. J., Kelly, J. W., and Colon, W. (1995) *Biochemistry* **34,** 13527–13536
16. Sunde, M., and Blake, C. C. F. (1998) *Q. Rev. Biophys.* **31,** 1–39
17. Colon, W., and Kelly, J. W. (1992) *Biochemistry* **31,** 8654–8660
18. Stevens, F. J., and Argon, Y. (1999) *Immunol. Today* **20,** 451–457
19. Yancey, P. H., and Somero, G. N. (1979) *Biochem. J.* **183,** 317–323
20. Garcia-Perez, A., and Burg, M. B. (1991) *Physiol. Rev.* **71,** 1081–1115
21. Burg, M. B., and Peters, E. M. (1998) *Am. J. Physiol.* **274,** F762–F765
22. Glenner, G. G., Ein, D., Eanes, E. D., Bladen, H. A., Terry, W., and Page, D. L. (1971) *Science* **174,** 712–714
23. Ronco, P. M., Aucouturier, P., and Mougenot, B. (1997) in *Diseases of the Kidney* (Schrier, R. W. and Gottschalk C. W. ed) Vol III, 6th Ed., pp. 2129–2174, Little, Brown, and Co., Boston
24. Kyle, R. A., and Gertz, M. A. (1995) *Semin. Hematol.* **32,** 45–59
25. Striker, L. J. M. M., D'Amico, G., Preud'bomme, J. L., and Striker, G. E. (1994) in *Renal Pathology* (Tisher, C. C. and Brenner, B. M., ed) Vol. II, 2nd Ed, pp. 1442–1490, J. B. Lippincott Co., Philadelphia
26. Marsh, D. J., and Knepper, M. A. (1992) in *Renal Physiology* (Windhager, E. E., ed) pp. 1317–1347, Oxford University Press, New York
27. Burg, M. B., Kwon, E. D., and Peters, E. M. (1996) *Kidney Int.* **50,** (suppl.) S100–S104
28. Timasheff, S. N. (1998) *Adv. Protein Chem.* **51,** 355–432
29. Wang, A., and Bolen, D. W. (1997) *Biochemistry* **36,** 9101–9108
30. Gekko, K., and Morikawa, T. (1981) *J. Biochem.* **90,** 39–50
31. Arakawa, T., and Timasheff, S. N. (1983) *Arch. Biochem. Biophys.* **224,** 169–177
32. Coelho-Sampaio, T., Ferreira, S. T., Castro, E. J., Jr., and Vieyra, A. (1994) *Eur. J. Biochem.* **221,** 1103–1110

33. Xie, G., and Timasheff, S. N. (1997) *Protein Sci.* **6,** 211–221
34. Tatzelt, J., Prusiner, S. B., and Welch, W. J. (1996) *EMBO J.* **15,** 6363–6373
35. Pace, C. N. (1986) *Methods Enzymol.* **131,** 266–280
36. Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989) *Anal. Biochem.* **177,** 244–249
37. Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry* **27,** 8603–8608
38. Dong, A., Huang, P., and Caughey, W. S. (1990) *Biochemistry* **29,** 3303–3308
39. Dong, A., Randolph, T. W., and Carpenter, J. F. (2000) *J. Biol. Chem.* **275,** 27689–27693
40. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) *Cell* **73,** 1055–1058
41. Harper, J. D., and Lansbury, P. T., Jr. (1997) *Annu. Rev. Biochem.* **66,** 385–407
42. Dong, A., Prestrelski, S. J., Allison, S. D., and Carpenter, J. F. (1995) *J. Pharmacol. Sci.* **84,** 415–424
43. Stevens, F. J., Myatt, E. A., Chang, C. H., Westholm, F. A., Eulitz, M., Weiss, D. T., Murphy, C., Solomon, A., and Schiffer, M. (1995) *Biochemistry* **34,** 10697–10702
44. Yang, D. S., Yip, C. M., Huang, T. H. J., Chakrabartty, A., and Fraser, P. E. (1999) *J. Biol. Chem.* **274,** 32970–32974
45. Davis, D. P., Raffen, R., Dul, J. L., Vogen, S. M., Williamson, E. K., Stevens, F. J., and Argon, Y. (2000) *Immunity* **13,** 433–442
46. Sluzky, V., Tamada, J. A., Klivanov, A. M., and Langer, R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9377–9381
47. Carpenter, J. F., Kendrick, B. S., Chang, B. S., Manning, M. C., and Randolph, T. W. (1999) *Methods Enzymol.* **309,** 236–255