Stimulation of Insulin Fibrillation by Urea-induced Intermediates*

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Fibrillar deposits of insulin cause serious problems in implantable insulin pumps, commercial production of insulin, and for some diabetics. We performed a systematic investigation of the effect of urea-induced structural perturbations on the mechanism of fibrillation of insulin. The addition of as little as 0.5 M urea to zinc-bound hexameric insulin led to dissociation into dimers. Moderate concentrations of urea led to accumulation of a partially unfolded dimer state, which dissociates into an expanded, partially folded monomeric state. Very high concentrations of urea resulted in an unfolded monomer with some residual structure. The addition of even very low concentrations of urea resulted in increased fibrillation. Accelerated fibrillation correlated with population of the partially folded intermediates, which existed at up to 8 M urea, accounting for the formation of substantial amounts of fibrils under such conditions. Under monomeric conditions the addition of low concentrations of urea slowed down the rate of fibrillation, e.g. 5-fold at 0.75 M urea. The decreased fibrillation of the monomer was due to an induced non-native conformation having significantly increased a-helical content compared with the native conformation. The data indicate a close-knit relationship between insulin conformation and propensity to fibrillate. The correlation between fibrillation and the partially unfolded monomer indicates that the latter is a critical amyloidogenic intermediate in insulin fibrillation.

Amyloid deposition of proteins has been found to lead to various diseases (1–4). Typical amyloid protein deposits consist of linear fibrils 60–130 Å wide and 1–6 microns long (5) with cross-β structure (6, 7) and exhibiting birefringence of bound Congo red under polarized light (8). The ability to form fibrils is independent of the origin, structural class, or function of the protein or polypeptide (9–12); in addition, non-amyloidogenic, stable globular proteins have also been observed to form fibrils on destabilization (13–18). These observations have led to a common belief that a conformational switch from the native conformation is responsible for fibrillation (2, 19–23). This is supported by the enhanced propensity for fibrillation under destabilizing conditions that result in the buildup of partially folded intermediates, such as mutations (24, 25), cosolvents (26), temperature (27), or pH (27, 28).

Insulin is a 51-residue hormone that may exist as a mixture of hexamer, tetramer, dimer, and monomer in solution, depending on the conditions. The protein is hexameric in the presence of zinc at neutral pH, monomeric in 20% acetic acid (pH 2), and dimeric in 20 mM HCl (pH 2.0). Insulin forms amyloid-like fibrils (29, 30) that pose a variety of problems in its biomedical and biotechnological applications (especially insulin pumps). Amyloid deposits of insulin have been observed both in patients with type II diabetes and in normal aging, as well as after subcutaneous insulin infusion and after repeated injection (31, 32). A number of investigations have been undertaken to elucidate the mechanism of insulin fibrillation (29–34). It has been proposed that insulin fibrillation occurs through the dissociation of oligomers into monomers and that the monomer undergoes a structural change to a conformation having strong propensity to fibrillate (29–31, 35–37).

Insulin fibrillation is accelerated by low to moderate concentrations of Gdn·HCl at physiological pH due to hexamer dissociation (38). The predominant species under these conditions was a partially folded monomer, which was present to >6 M Gdn·HCl. Similar effects are observed when starting from monomeric insulin, due to the presence of a partially folded conformation. Thus a minimum pathway for insulin fibrillation is hexamer → monomer → partially unfolded monomer → fibrils (38). In this study we have investigated the relationship between conformation of insulin induced by urea and the propensity to fibrillate, starting both from hexamer (pH 7.4) and monomer. Fibril formation was followed with a Thioflavin T binding fluorescence assay (39) and structural changes were observed using optical spectroscopy, size-exclusion chromatography, and small angle x-ray scattering. The results show a strong correlation between the association and conformational states of insulin and its propensity to fibrillate, and significant differences to the effects of Gdn·HCl. The results, in conjunction with earlier studies on insulin fibrillation (38), enhance our understanding of the relationship between conformational change and fibrillation of insulin.

EXPERIMENTAL PROCEDURES

Materials

Human insulin with zinc was kindly provided by Novo Nordisk, Copenhagen, Denmark. All chemicals were analytical grade, and were from EM Sciences or Sigma. Thioflavin T was obtained from Fluksa.

Preparation of Samples

Stock solutions of human insulin, 2–10 mg/ml, were prepared fresh before use in 0.025 M HCl, pH 1.6. For experiments starting with hexameric insulin, aliquots of 1 M HEPES buffer were added to obtain a final concentration of 50 mM HEPES and adjusted to pH 7.4 using NaOH. For studies of monomeric insulin, a stock solution was freshly prepared by dissolving human insulin in 20% acetic acid (pH 2.0). The concentrations of Gdn·HCl were determined by titration with NaOH.

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The abbreviations used are: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; FTIR, Fourier transform infrared; ThT, thioflavin T; SEC, size-exclusion chromatography; SAXS, small-angle X-ray scattering.
efficient of 24,420 at 420 nm. ThT was stored at 4 °C, protected from light. The effect of urea on conformation and fibrillation was studied by mixing aliquots from the insulin stock solutions to final concentrations of 2 mg/ml protein, 50 mM HEPES, 20 μM ThT, and urea concentration from 0–6 M. In the case of the studies at pH 2, aliquots were added to each sample to reach a final concentration of 20% acetic acid, 2 mg/ml human insulin, 20 μM ThT, and urea varying from 0–6 M.

Methods

Kinetics of Fibrillation—ThT fluorescence was monitored for each sample in a 96-well plate in a Fluoroskan Ascent CF fluorescence plate reader (Lab Systems) at 37 or 40 °C and shaking at 760 rpm. The plate was sealed with Mylar plate seals (Thermo Labsystems). The excitation wavelength was 444 nm and the emission measured at 485 nm. Plates were continuously shaken for the entire period of study at a rotation diameter of 1 mm, and the integration period was fixed at 20 ms for each well. Five replicates, corresponding to five wells for each sample, were used to minimize well-to-well variation. Results from >3 similar profiles were averaged for the final results at each urea concentration. The kinetic profiles were analyzed by curve-fitted using Sigmaplot software (30).

Circular Dichroism—CD spectra were taken at 25 °C on an AVIV 60DS circular dichroism spectrophotometer (Aviv Associates, Lake-wood, NJ). Aliquots from stock solutions were mixed to a final concentration of 2 mg/ml protein, and urea varying from 0–6 M. Protein, and buffer contributions from buffer, solutions without protein were prepared in the same manner as above, and their spectra subtracted from the protein spectra. Samples were incubated for 2 h before collecting the spectra. Both near- and far-UV spectra were recorded using a step size of 0.5 nm and a bandwidth of 1.5 nm. In the far-UV region spectra were recorded in a 0.01-cm cell, and for the near-UV experiments a cell of 0.5-cm path length was used.

Analysis of Spectroscopic Data (Phase Diagrams)—The phase diagram method analysis of spectroscopic data is extremely sensitive for the detection of intermediate states (40–43). The essence of the phase diagram method is to build up the diagram of I1 versus I2, where I1 and I2 are the spectral intensity values measured at wavelength A1 and A2 under different experimental conditions for a protein undergoing structural transformations. The non-linearity of this function reflects the sequential character of structural transformations, where each linear portion of the I(λ1) vs I(λ2) plot describes an individual all-or-none transition.

Small Angle X-ray Scattering—Small angle x-ray scattering measurements were made using the SAXS setup on Beam Line 4–2 at Stanford Synchrotron Radiation Laboratory, as described previously (38). The values of radii of gyration (Rg) were calculated according to the Guinier approximation (44); In(Q) = In(0) - Rg2 Q2/3 where Q is the scattering vector given by Q = 4π sin θ /λ (θ is the scattering angle, and λ = 0.154 nm). The forward scattering amplitude I(Q) = A/ Q − 1/2 is proportional to the square of the molecular weight of the molecule (44). I(0) for a pure dimer sample will therefore be twice that for a sample with the same number of monomers since each dimer will scatter four times as strongly, but there will be half as many as in the pure monomer sample.

Size Exclusion Chromatography—Size exclusion chromatography was performed using a Superose 12 HR 10/30 column from Amersham Biosciences (exclusion limit 2 x 106 M-1). The column was run on a FPLC GP 250-P500 (Amersham Biosciences) instrument at a flow rate of 0.4 ml/min with detection at 280 nm. Human insulin, 2 mg/ml was incubated in 50 mM HEPES pH 7.4, with and without urea for 2 h at 25 °C. Aliquots of 200 μl of each sample were loaded on the column, which was equilibrated with 50 mM HEPES buffer containing the desired concentration of urea prior to loading of sample. For experiments involving variation in insulin concentration, 0.5–10 mg/ml of insulin was incubated in 2.0 M urea, 50 mM HEPES pH 7.4. Aprotinin (M, 6.5 kDa) and cytochrome c (M, 12.3 kDa) were run as markers in the presence of 0.5 M urea, 50 mM HEPES pH 7.4. Since no significant changes in these proteins have been observed in 0.5 M urea, these markers were assumed to be globular under these conditions. The observed elution volume of 15.0 and 14.0 ml (data not shown) for aprotinin and cytochrome c is consistent with their size.

UV Absorbance Studies—Human insulin, 2 mg/ml was prepared and incubated in wells in the fluorescence plate reader as discussed above, however, ThT with a final concentration of 20 μM was added to only one of the five wells and was taken as a reference to monitor formation of
fibrils. Once the transition into fibrils was complete the contents from each of the wells were transferred to 0.5-ml centrifuge tubes and centrifuged at 14,000 rpm for 15 min. The supernatant was carefully removed, and the absorbance for protein or ThT measured. For protein, 50 μl of the supernatant was dissolved in 950 μl of 25 mM HCl, 0.1 M NaCl pH 2.0. The percentage of protein left in the supernatant was calculated by taking the absorbance of 2.0 mg/ml protein in 50 mM HEPES pH 7.4, processed in a similar manner (50 μl in buffer), as 100%. For samples in 20% acetic acid the pellet obtained as above was dissolved in 600 mM NaOH and the absorbance read at 290 nm.

Transmission Electron Microscopy—Aliquots of 5 μl of insulin fibrils grown in microtiter plates without ThT at 2 mg/ml insulin were diluted once and 5 μl of resulting solution placed onto carbon grids coated with Formvar (Ted Pella Inc.) and left for 1–5 min. The grid was washed five times with nano-pure water and five times with 1% uranyl acetate for negative staining and left to dry at room temperature. The specimens were viewed and images recorded with a Philips 208 electron microscope operated at 80 kV using Gatan digital micrography software.

Acrylamide Quenching—Tyrosine fluorescence quenching experiments were carried out on insulin solutions at 25°C. Insulin stock solution of 2 mg/ml was prepared in 20% acetic acid. Aliquots to give a final concentration of 0.1 mg/ml (~1 μM) were added to the solutions containing varying urea concentration. Samples were incubated for 2 h before taking the fluorescence measurements. For each sample aliquots from an acrylamide stock (4.0 M) were added with a Hamilton syringe to reach a final concentration of acrylamide varying from 0–175 mM and the fluorescence measured immediately. The samples were excited at 276 nm, and emission was monitored at 301 nm.

RESULTS

Insulin fibrillation has been shown to be very sensitive to environmental conditions (30, 38, 45). Various biophysical methods were applied to study changes in the conformation...
induced by urea both for hexameric insulin at pH 7.4 and monomeric insulin in 20% acetic acid.

**Urea-induced Quaternary, Tertiary, and Secondary Structure Changes in Insulin at pH 7.4**—Information about the quaternary and tertiary structure of insulin were obtained by monitoring changes in the near-UV CD region (46). A broad negative peak centered at 276 nm was observed for native hexameric protein. Since there are no tryptophan residues in

![Figure 3](http://www.jbc.org/Downloaded from)

**Fig. 3. Changes in the association state and compactness of insulin as a function of urea.** Absorbance profiles from size exclusion chromatography experiments with insulin (2 mg/ml) in varying concentrations of urea, pH 7.4. A, changes in the oligomeric state and compactness: the numbers represent the urea concentration. B, SEC profiles obtained after incubating 0.5, 2.0, and 10.0 mg/ml insulin in 2.0 M urea for 2 h prior to injection onto the column.
insulin, the peak reflects the contributions of tyrosine. Fig. 1A represents some typical spectra depicting changes in the shape and intensity of the profiles upon addition of varying concentration of urea. Insignificant changes between 0 and 2 M urea were apparent followed by a major transition between 2 and 4.75 M urea (midpoint ∼3.5 M). Above 5 M gradual changes were observed up to 7.5 M urea. The spectrum for 7.75 M urea exhibits residual tertiary structure. These observations reflect a constantly changing environment of tyrosines with increasing urea concentration, with a broad transition centered at ∼3.5 M urea (Fig. 1C).

Far-UV CD spectra obtained with various urea concentrations are shown in Fig. 1B. For native insulin, minima at 222 nm and 209 nm, characteristic of α-helix, were observed. The changes in the secondary structure of insulin upon addition of increasing concentrations of urea were reflected as marked changes in the shape and intensity of the spectra. However, up to 3.0 M urea significant changes were observed only in the vicinity of 209 nm; large changes around 222 nm region were observed only after the addition of 4.0 M and higher urea concentrations. The spectrum obtained at 7.0 M urea does not resemble that of an extensively unfolded protein, and can be attributed largely to the weak chaotropic effect of urea and also to the resistant super-secondary structure of the insulin B chain (45, 53). The ellipticity at 222 nm shows a broad transition beginning at 3.0 M urea and extended above 7 M (Fig. 1C). This transition is at significantly higher urea concentrations than that observed at θ276 indicating that the changes in the quaternary and tertiary structure are uncoupled from those of the secondary structure, and reflecting the buildup of partially unfolded intermediate conformations.

CD data have been traditionally analyzed in terms of fraction folded/unfolded or θ_n m versus denaturant concentration (as in Fig. 1C), but phase diagrams can be very informative in data analysis, and have been used to resolve complex structural changes from both fluorescence and CD data (42, 43). Fig. 2A represents the phase diagram obtained by plotting the ellipticity at 276 nm against that at 251 nm. These wavelengths were chosen based on the large variations of ellipticity in their vicinity (Fig. 1A). The phase diagram shows four linear segments from 0–0.75, 0.75–1.75, 1.75–4.25, and 4.25–7.25 M urea. Each linear segment represents an all-or-none transition between two different conformations (42, 43), and thus there are at least five structurally distinct species that become populated as a function of increasing urea concentration.

For far-UV CD, the phase diagram was obtained by plotting ellipticity at 222 nm versus that at 210 nm and is shown in Fig. 2B. Five sets of different conformational species were observed as indicated by the linear segments, ranging from 0–1.0, 1.0–3.0, 3.0–4.0, and 4.0–7.0 M urea. This implies the existence of
five species with differing secondary structure over the range of 0–7.0 M urea. From comparison of the two phase diagrams, the regions of 0–1.0 and 4.25–7.5 M urea represent conditions under which there are simultaneous changes in both secondary and tertiary structure. The two other conformations at intermediate urea concentrations seem to exhibit independent secondary and tertiary structural rearrangements.

Changes in the Association State of Insulin Induced by Urea at pH 7.4—Insulin samples were incubated in the presence and absence of varying concentrations of urea for 2 h before injection onto a Superose S12 column pre-equilibrated with the corresponding concentration of urea. The results from SEC are summarized in Fig. 3A. For native insulin, an elution volume of 6.1 ml, corresponding to the hexamer was observed. The addition of 0.5 M urea to insulin led to a broad elution peak spanning 12.4–14.2 ml. The position of this peak lies between those of the insulin hexamer and dimer: the broadness of this peak indicates the presence of more than one oligomeric species existing in an equilibrium mixture with a significantly faster dissociation/association compared with the time of chromatographic separation. Only small shifts were observed in the elution peak of insulin as higher urea concentrations were added, indicating that the dissociation process or oligomeric state of insulin was not significantly influenced by urea. The maximum of intensity of the elution peak was at 12.4 ml in the case of 0.5 M urea and was observed to undergo a small shift to 13.5 ml at 7.5 M urea. Thus the data suggest a rapid (on the time scale of the chromatography) equilibrium between two or more species; from what is known about the assembly process of insulin (see below) the simplest situation would be an equilibrium between hexamer and dimer, heavily weighted in favor of the dimer. The elution volume for the unfolded monomer is around 14.5 ml, and for the native (compact) dimer it is 13.4 ml. At high urea concentrations the unfolded monomer will be present (see below) and appears to have an elution volume similar to that for the compact dimer.

The elution profiles obtained with varying concentrations of insulin at constant 2.0 M urea (Fig. 3B) failed to resolve the broad peak into components and resulted only in a change in the elution volume. This is consistent with a shift in the equilibrium toward the hexamer with increase in insulin concentration.

Urea-induced Changes in the Size and Packing Density of Insulin—Small-angle x-ray scattering has been used to gain information about the ensemble distribution of molecular size, shape, compactness and packing (globularity) and oligomeric state (44, 47, 48). Insulin, 4 mg/ml, was incubated for 2 h at pH 7.4 in the presence and absence of the desired concentration of urea before taking the SAXS measurements. This was the lowest concentration of insulin that could be studied due to instrumental limitations. A compact bell-shaped Gaussian curve at lower angles, characteristic of a compactly folded globular protein was observed for native hexameric insulin at pH 7.4 in the Kratky plot (Fig. 4). At 1 M urea a decrease in the intensity of signal at lower angles suggests a decrease in the concentration of the large compact hexamer and population of smaller species. The intensity of the peak at lower angles shows a further decrease with increasing urea concentration. Surprisingly, the profile obtained at 7.0 M urea (Fig. 4, inset) retains a bell-shaped Gaussian curve at lower angles indicating the existence of a species with residual compactness even at very high urea concentrations. Dissociation of the hexamer into smaller oligomers and monomers is expected to be accompanied by changes in the position of the scattering maximum toward the high scattering angles. The lack of such shift could be explained by the fact that SAXS data are weighted heavily by hexamers, whereas dimers and monomers have only a small contribution to the overall scattering. Furthermore, this contribution of dimers and monomers is substantially diminished by the increase in the background scattering from the urea (i.e. due to the decrease in contrast). Since the protein concentration for the SAXS measurements was much higher than that for the SEC experiments (due also to dilution on the column), the equilibrium will be shifted to higher levels of higher oligomers in the SAXS measurements, compared with the chromatography experiments.

Comparative Analysis of SEC Data—It is not possible to unambiguously ascertain the constituent oligomeric species from insulin dissociation as a function of urea concentration from the SEC profiles. However in other studies (46) it has been shown that insulin assembles into hexamer through association of three dimers. Also in our previous studies (38) we observed the dimer to be the predominant dissociation product of the hexamer, retaining stability up to ~3 M Gdn-HCl. Thus it is more plausible to assume that hexameric insulin dissociates
predominantly into dimeric species with increasing urea concentration. This assumption is supported by data shown in Fig. 5. The plot of the position of chromatographic peak against urea concentration shows two transitions. The first transition is completed by 3.5 M urea, whereas the second one starts at 5.5 M urea. A region between 3.5–5.5 M urea exhibiting no significant changes separates the transitions. Another interesting feature was observed by comparing the widths of the SEC elution peaks. At lower concentrations of urea the peaks were observed to be broad indicating the existence of more than one species in fast equilibrium; at higher concentrations the peaks were narrow with increased intensity indicating the existence of a single predominant species. The plot of elution peak width against urea concentration (Fig. 5) again shows a stable intertransition region between 3.5–5.5 M urea flanked by two similar transitions as discussed above. These observations indicate that insulin dissociates predominantly into a species that is stable up to a fairly high concentration of urea (5.5 M). Since other oligomeric species (tetramer or trimer) are very transient and unstable the findings clearly indicate that insulin dissociates predominantly into a dimeric form under these conditions. Above 5.5 M urea the changes are more consistent with the unfolding of monomeric insulin as discussed in the next section.

**Insulin Association State from Near-UV CD Difference Spectroscopy**—Near-UV CD difference spectroscopy has been used to predict the association states of insulin under various structure perturbing conditions (46). Three of the four tyrosines present in insulin are located on the surface, and dimerization shields at least two of these during dimer interface formation (49). Thus, near-UV CD difference spectra, since they reflect changes in the environment of the tyrosines, could provide useful information about the oligomeric state. The spectrum with the deepest negative band around 276 nm in Fig. 6, represents the difference spectrum (relative to monomeric insulin) of native insulin at pH 7.4 and corresponds to the difference spectrum of the hexamer of insulin (46). At 6.75 M urea the difference spectrum exhibits near zero signal suggesting monomeric insulin. For the 7.75 M sample a difference spectrum with a positive band around 276 nm corresponds to that for a partially unfolded monomeric form.
FIG. 8. The effect of urea on monomeric insulin, monitored by circular dichroism. Panel A shows selected near-UV CD spectra. Panel B shows selected far-UV CD spectra, and panel C shows the urea dependence of ellipticity at 222 (open triangles), 251 (filled circles), and 276 nm (open circles). Conditions were: insulin 2.0 mg/ml, 20% acetic acid, pH 1.6, 25 °C. Each spectrum is the average of two independently collected spectra. In panel A, the urea concentrations at 270 nm starting from bottom are, 0.25, 0.75, 3.25, 0.0, 2.25, 4.25, 7.25 M. In panel B, the urea concentrations are, in order of decreasing ellipticity at 222 nm, 0.75, 0.5, 1.25, 0.0, 3.25, 5.5, 6.75 M.
Kinetics of Fibrillation for Hexameric Insulin in the Presence of Urea—Conditions that lead to the population of partially folded intermediates in general lead to enhanced fibrillation of proteins (50, 51). In order to correlate the propensity of insulin to fibrillate with the various conformational states characterized above, the fibrillation of insulin at pH 7.4 was monitored as a function of urea concentration, starting with the hexamer.

Solutions of 2 mg/ml insulin were incubated at pH 7.4 and 40 °C with agitation. Similar patterns were observed at 37 and 25 °C except for slower kinetics at the lower temperatures (data not shown). The kinetics of fibrillation were monitored by Thioflavin T fluorescence in the presence and absence of urea, and representative results are given in Fig. 7. Similar kinetics were observed when fibrillation was monitored by light scattering (data not shown), indicating that there was no significant buildup of oligomeric intermediates prior to formation of fibrils. The curves in Fig. 7A exhibit a sigmoidal pattern with an initial lag phase, an exponential growth phase and a final plateau phase, corresponding to nucleation, extension, and equilibrium phases, respectively. Fig. 7B shows the lag times calculated from curve fitting as a function of urea concentration. The native hexamer had a lag time of 17.5 h. The addition of 0.5 M urea resulted in a decrease in the lag time to 9.4 h, indicating an enhanced rate of fibrillation. The faster nucleation for the dissociated species is consistent with rate-limiting dissociation of the hexamer in fibrillation under physiological conditions. The lag time gradually decreased as the concentration of urea was increased until it reached 4.4 h at 2.0 M urea. Above 2.5 M urea no significant further changes in lag time were observed. Thus, the kinetics show an increased rate of fibrillation (nucleation) between 0 and 2.5 M and a relatively constant lag time from 2.5 to 7.0 M urea (Fig. 7B). The rate of fibril elongation is only very slightly increased by the presence of urea.

The amount of fibrils formed, as a function of urea, as measured by the final ThT fluorescence signal, did not show a significant change as the denaturant concentration increased, Fig. 7B. This observation was supported by quantification of the amount of protein in the supernatant and pellet at the end of the fibrillation as a function of urea concentration (see “Experimental Procedures”). The results show that after incubation at 37 or 40 °C, pH 7.4, ~97% of insulin is present in the insoluble fibrils, at all urea concentrations. Even at 7.0 M urea only 4% protein was found to remain soluble.

Comparison of the rate of fibril formation with the changes in structure suggests that the dissociation of the hexamer has a profound effect on the rate of insulin fibrillation, and is, in fact, the rate-limiting step. This is supported by the decrease in lag time between 0 and 2 M urea, which is consistent with the data of Fig. 5, showing the conversion of hexamer to dimer over this range. Further, the rate of fibrillation plateaued at 4.5 M urea; conditions where the predominant species is the expanded monomer (see Fig. 13). Thus the maximal rate of fibrillation above 4.5 M urea is consistent with the expanded monomer conformation (see below) being the critical amyloidogenic intermediate in fibrillation, although it is likely that the expanded dimer (see below) also is comparably amyloidogenic.

Effects of Urea on Acrylamide Quenching of Monomeric Insulin—Further proof of the structural changes taking place in the monomer with increasing urea concentrations was obtained from fluorescence quenching experiments (54). Fig. 10 shows the Stern-Volmer plots obtained at various urea concentrations. A decrease in the slope between 0.25 and 1.5 M urea suggests restricted accessibility for tyrosines under these conditions. As three of the four tyrosines of insulin are located in helices, the observation of decreased tyrosine accessibility is consistent with the far-UV CD data that shows a large increase in helical content in this range of urea (Fig. 8B). At 3.0 M the slope of the Stern-Volmer plots reflect tyrosine accessibility comparable to that of the control (no urea) and at 4.0 and 6.0 M the slope increases, showing an increase in the accessibility of the tyrosines due to unfolding of the molecule.

Small Angle X-ray Scattering of Monomeric Insulin—Fig. 11 represents the Kratky plots obtained in 20% acetic acid and different urea concentrations. The profile for the insulin monomer shows a broad bell-shaped Gaussian curve, indicative of a compact protein, but not as tightly packed as in the native hexamer (46). The addition of increasing urea concentration does not result in any significant change in the intensity or shape of the profiles. At urea concentrations as high as 6.0 M the plot shows retention of compactness and packing density in the form of bell-shaped curve similar to those of the control. The scattering intensity Iq can be used to determine changes in the molecular mass; i.e. association state, of the sample as a function of urea concentration. No evidence for any association of monomer was observed, i.e. Iq remained constant.

Fibrillation of Monomeric Insulin—Fig. 12A shows representative data for the kinetics of monomeric insulin fibrillation, monitored by the characteristic increase in ThT fluorescence intensity. Surprisingly, low concentrations of urea caused a substantial decrease in the rate of insulin fibrillation. For example, at 1.0 M urea the lag was almost 5 times longer compared with the monomer in the absence of denaturant.
Fig. 9. Phase diagrams for monomeric insulin from near- and far-UV CD data. A, changes in the near-UV CD. B, changes in the far-UV CD and C, simultaneous changes in both far- and near-UV CD. See legend to Fig. 2 for details.
Between 2.5 and 3.5 M urea the lag time was comparable to that of the control. Above 3.5 M urea the lag time showed a progressive increase with increasing urea concentration. The variation in ThT fluorescence, along with the midpoints of the transition versus urea concentration as shown in Fig. 12 reveal a region from 0 to 1.5 M urea of lower fibril yield accompanying the slower fibrillation kinetics as compared with control. Thus, this conformationally distinct state renders insulin resistant toward fibrillation. The reduced rates of fibrillation at higher concentration of urea could be attributed to the decreased population of partially folded intermediates and the solubilizing effect of urea.

Electron microscopy was used to compare the morphology of insulin fibrils in the presence and absence of urea (data not shown). The fibrils had the same width (10–12 Å) but varied significantly in their length and also the degree of clumping. Starting with both hexamer and monomer, the presence of increasing urea decreased the length of the fibrils, led to increased clumping of fibrils, and increased the crystalline appearance of the fibrils.

**DISCUSSION**

In the absence of denaturants there is little direct evidence for the population of partially folded intermediates in the fibrillation of insulin, suggesting that such intermediates normally have short lifetimes. The present investigation was undertaken to determine if partially folded conformations of insulin could be populated by low to moderate urea concentrations, and to determine the relationship between conformation and fibril formation. The starting states of insulin were the hexamer at pH 7.4, and the monomer in 20% acetic acid (46, 55). Pathological protein aggregation and fibril formation is believed to require the critical intermediacy of partially folded amyloidogenic conformations (51, 56). In contrast to Gdn-HCl, urea, which is a weaker chaotrope, induces more subtle changes in the conformation and fibrillation pattern of insulin (38, 57).
The Effects of Urea on Insulin at pH 7.4—The changes in the near- and far-UV CD and SEC profiles were used to calculate the fractions of the different states (Fig. 13A) as a function of urea concentration. A summary of the changes induced by urea in insulin at pH 7.4, as monitored by the different techniques used in this study, is given in Fig. 13 and Scheme 1,

$$
H \quad \leftrightarrow \quad D \quad \leftrightarrow \quad D_{\text{exp}} \\
M \quad \leftrightarrow \quad M_{\text{exp}} \quad \leftrightarrow \quad M_U
$$

**Scheme 1**

where H represents hexamer, D is dimer, M is monomer, and the subscript exp is expanded, and U is unfolded.

Scheme 1 is based on the following observations: dissociation of hexamer occurs at very low concentrations of urea (< 0.5 M), as observed by SEC. The initial changes in insulin tertiary structure at low urea concentrations occur with dissociation of hexamer into dimers within a small range of urea concentrations. The transition, characterized by a C_m of 0.75 M urea, leads to a mixture of predominantly compact and expanded dimer. In the region between 3.0 and 5.5 M urea, the major species are dimers based on SEC. On the other hand the loss of far-UV CD around 5.5 M urea indicates the presence of partially unfolded or expanded conformations. Above 5.5 M urea only monomers are present as shown by near-UV CD difference spectra. CD spectra obtained at 7.0 M urea show considerable secondary structure and therefore the protein is not completely unfolded under these conditions, which is consistent with the observation of some remaining globular structure in the Kratky plot (Fig. 5) at the highest urea concentration. This also explains non-overlapping of changes in secondary structure and hydrodynamic dimensions from SEC data that usually occur simultaneously (38).

**Correlation of Partially Folded Intermediates and Fibrillation from Hexameric Insulin**—The data at pH 7.4 reveal the existence of a total of five different conformations of insulin, depending on the urea concentration. The hexamer initially dissociates into the compact dimer that undergoes partial unfolding to the expanded form followed by dissociation into monomers that, in turn, retain some structure at fairly high
Fig. 13. Urea dependence of structural changes in insulin. Panel A, fractional changes in various parameters; $\theta_{208}/\theta_{222}$, open circles; first transition in SEC elution volume, filled circles; $\theta_{222}$, filled inverted triangles; $\theta_{208}$, inverted triangles. Panel B, fractions of each insulin species at pH 7.4, calculated from SEC and CD data, native hexamer (H$_N$), compact dimer (D$_C$), expanded dimer (D$_{exp}$), unfolded monomer (M$_U$). The fraction of D/D$_{exp}$ was calculated from the difference between the curve representing the decrease in fraction of native and the curve representing fraction unfolded from circular dichroism. The dotted line represents the fraction of the dimer obtained from SAXS data. Panel C, fractions of insulin monomer in 20% acetic acid: compact monomer (M$_C$), unfolded (M$_U$), and expanded monomer (M$_{exp}$) were calculated from CD data.
concentrations of urea before substantial unfolding takes place. The strongest evidence for partially folded/unfolded structures is provided by the circular dichroism data, which indicates the existence of a complex set of conformationally distinct states in the phase diagrams at pH 7.4. Analysis of the CD data in the form of phase diagrams makes information available in much greater detail (Fig. 2) relative to the traditional analysis of CD data (Fig. 1C). At least four non-native conformations/states were observed for insulin (pH 7.4) over the whole urea range.

Although the correlation of specific insulin conformations with fibrillation is complicated because of the concurrent presence of multiple states (Fig. 1B), as well as the fibril-dissolving effect of very high urea concentrations, our data clearly show that fibrillation arises from a partially folded conformation, namely the expanded monomer. Thus we conclude that the partially folded monomer is responsible for fibrillation at both neutral and low pH.

Previous studies have established that insulin self-assembly involves the initial formation of dimers, three of which then associate to form the hexamer. Structural studies have shown that there are two different interfaces, monomer-monomer to form dimer, and dimer-dimer to form hexamers. Our data are consistent with the particular stability of the dimer and hexamer states, reflecting the stronger interfacial interactions for these types of assemblies. The fact that the dimeric species are far more populated in urea indicates that the monomer-monomer interface is much stronger than that for the dimer-dimer interface.

**Correlation of Partially Folded Intermediates and Fibrillation from Monomeric Insulin**—The complexity of insulin aggregation at pH 7.4, due in part to the contribution of several oligomeric species, makes it difficult to unambiguously ascertain the aggregation pathway. In order to obtain a better understanding of insulin aggregation and fibrillation we carried out studies on the monomeric form of insulin in 20% acetic acid (46, 55). CD, acrylamide fluorescence quenching and SAXS studies demonstrate the existence of multiple conformations and varying degrees of unfolding as a function of urea concentration under these conditions. The relative concentrations of the various conformational states as a function of urea are presented in Fig. 13C. At low concentrations, between 0 and 1.5 M urea, a significant increase in helical content of insulin was observed by CD, which was corroborated by quenching studies showing reduced accessibility of tyrosines; the majority of which lie in the helix region. Within this concentration range of urea a significant decrease in the kinetics of insulin fibrillation was observed. This decrease in the rate of nucleation is attributed to the change of insulin into a more stable conformation with increased helicity under these conditions. The SAXS I (0) values indicate that association of monomers is not involved in this process, and the Kratky plot suggests an increase in compactness. The origin of this substantial conformational change in the insulin monomer at low urea concentration is not clear, but probably represents the net result of a combination of competing interactions between the protein, water and urea. Between 1.5 and 4.5 M urea insulin exists predominantly as the expanded monomer, (Fig. 13C) with substantially faster fibrillation than the control (no urea). Higher urea concentrations lead to a transition into the unfolded form, as indicated by near- and far-UV CD and acrylamide quenching. The CD phase diagrams show that there are five major monomeric conformations of insulin: the native monomer (M), a compact monomer with increased helix (M12), two forms of the expanded monomer (Mexp) and the unfolded monomer (M12). The data indicate that the latter is not fully unfolded, but retains some residual structure. The decreased fibrillation at high urea concentrations is due to two concurrent processes: the decreasing amount of expanded monomer (which means that the concentration of the amyloidogenic species is decreased, leading to slower kinetics), and the increasing concentration of urea, which disaggregates the fibrils. Despite these constraints, fibrils were observed up to 5.5 M urea, reflecting the low concentrations of expanded monomer present.

Interestingly, there are substantial differences between the effects of urea and Gdn-HCl on insulin conformation and fibrillation; it is possible that these reflect the increased ionic strength that occurs when Gdn-HCl is used as a denaturant. These differences are most apparent in the behavior of monomeric insulin (38), which shows the presence of the compact, helix-rich conformation that is slow to fibrillate, in the presence of urea but not Gdn-HCl. Other significant differences include the lack of resolution of intermediates in the presence of urea by SEC, the greater sensitivity of the hexamer dissociation to Gdn-HCl, the relatively much slower fibrillation of monomeric insulin in the presence of urea and the relative differences in the populations of different intermediates during fibrillation. No stable non-fibrillar aggregates were observed either in the presence or absence of denaturants.

This investigation demonstrates that fibrillation of hexameric insulin requires dissociation to monomer followed by conformational changes to form a critical amyloidogenic partially unfolded intermediate. Although urea was used in the present study, we believe that a similar situation will exist in the absence of the denaturant, with the caveat that the concentrations of the intermediate species will be significantly lower than in the presence of the destabilizing denaturant, leading to the observed slower fibrillation. Since fibrillation arises from a partially unfolded monomeric intermediate, which lacks significant tertiary structure and limited secondary structure, models for fibril structure, based on the three-dimensional structure of the insulin native state (21, 58–61), are unlikely to be correct. In fact, we believe that substantial unfolding of the native state is necessary in order to permit the required topological changes for the β-structure of the fibrils to occur.

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