Supporting Material – Perturbation of the stability of amyloid fibrils through alteration of electrostatic interactions

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Estimation of protein charge at various pH values

The charge of each insulin molecule (Q) and its estimated error were calculated for a range of pH values as the mean and standard deviation of a simulated set of 10,000 Q-pH curves using Matlab software package (MathWorks, MA, USA). These simulated curves were created by independently assigning each ionisable moiety of the protein a pK_a value (according to a Gaussian probability distribution function described by the mean and standard deviations presented for that moiety presented by Pace et al. (1)), and then calculating the resultant Q by summing the charge contributions of each moiety at a range of pH values. The contributions of arginine, histidine, lysine and the N-terminus were calculated as a function of their assigned pK_a according to Eq. 1. The contributions of aspartic acid, cysteine, glutamic acid, tyrosine and the C-terminus were calculated as a function of their assigned pK_a according to Eq. 2.

$$Q_c = \frac{10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \tag{1}$$

$$Q_c = \frac{10^{pH-pK_a}}{1+10^{pH-pK_a}} \quad (2)$$

To our knowledge this approach for estimating protein charge has not been used previously. However the dataset of Pace et al. (1) is the most recent and thorough assessment of the experimental variation in pKa values observed in the context of folded proteins. We therefore believe it is the best dataset upon which charge estimates in challenging systems can be made. In general support of our approach we note that an experimentally obtained proton titration curve for native soluble human insulin obtained by Tanford and Epstein (2), is within the predicted range for Q-pH curves for insulin obtained here.

Secondary structure of insulin determined by ATR-FTIR

In order to estimate the contribution of the different secondary structural elements in insulin fibrils at pH* 1.6 and 7.6 to the amide I' region of the infrared spectra, second derivatives of the spectra between 1600 and 1700 cm^{-1} were initially taken to identify component peak positions. The positions of the major minima from these analyses were subsequently used for fitting the unmodified spectra to a series of Gaussian functions (3). The heights, widths and positions of the Gaussian functions were varied iteratively to achieve the line of best fit using the OriginPro 7.5 software package (OriginLab, MA, USA). In Fig. S1 the lines of fit to the spectra are indistinguishable from the raw data. Integration of the Gaussian curves was used directly to determine the secondary structure content, the results of which are shown in the table in Fig. S1. The errors in percent secondary structure are $\sim 5\%$ (4).

Equilibrium dissociation of insulin fibrils in guanidinium thiocyanate

The chemical stability of insulin fibrils was determined following incubation of the fibrils in guanidinium thiocyanate for 48 h. In order to confirm that this incubation time is sufficient for the reaction to reach equilibrium, samples were also tested after incubation in denaturant for 24 h. Fibrils were added at a final concentration of 0.3 mg/ml to solutions containing various concentrations of GdnSCN and 30 mM glycine (pH 2.0), 30 mM acetate (pH 4.0), 30 mM citrate (pH 6.0) or 30 mM tris-HCl (pH 8.0). Solutions were mixed by vortexing and incubated at room temperature. Aliquots were removed from these samples after 24 h and 48 h and were

immediately centrifugated in a Beckman Optima TLX ultracentrifuge at $288,000 \times g$ for 40 min. The protein concentration in the supernatants was quantified using the Bradford assay, and used as a measure of the fraction of soluble protein. As shown in Fig. S2, the fraction of soluble protein is almost identical between 24 h and 48 h at the denaturant concentrations tested across the pH range 2.0 - 8.0, indicating that the dissociation process reaches equilibrium within 24 h.

Reversibility of the fibril dissociation reaction

To test the reversibility of the fibril dissociation reaction, and thus validate our use of the linear polymerization model to fit these data, 0.9 mg/ml insulin fibrils were incubated at either pH 11.0 (sample A), or pH 2.0 in the presence of 5 M GdnSCN (sample B) for 48 h to induce dissociation of the fibrils. Re-aggregation of both samples was initiated by a 3-fold dilution into 30 mM glycine buffer, pH 2.0. This yielded a final protein concentration of 0.3 mg/ml in both samples, with a final pH of 2.3 for sample A and a final denaturant concentration of 1.7 M for sample B (the pH of this solution remained unchanged at pH 2.0). Samples were incubated at room temperature and the soluble fraction of insulin determined at 0 h and 48 h as outlined in the methods. As shown in Figs. S3*A* and *B*, at 0 h both samples contained $> 95\%$ soluble protein (red diamonds). After 48 h, however, there were negligible quantities of soluble protein in both samples (Figs. S3*A* and *B*, blue inverted triangles) establishing the apparent reversibility of the dissociation reaction. Taken together, these results indicate that even under the highly denaturing conditions employed a very small population of fibrils remain resistant to the strongly alkaline conditions and the high concentration of GdnSCN and act as seeds to initiate fibril proliferation when the solution conditions are made more favourable for fibril elongation. Narimoto et al. (5) have demonstrated similar findings for the protein β_2-microglobulin.

The reversibility of the dissociation reaction was also examined by assessing whether fibrils could elongate under seemingly unfavourable, alkaline conditions. Here, 5% w/w insulin fibrils (formed at pH 2.0) were added into a solution of 0.3 mg/ml soluble insulin at pH 9.5. Previous experiments had shown that the adjustment to pH 9.5 of a solution containing only fibrillar insulin at a final protein concentration of 0.3 mg/ml resulted in the liberation of insulin molecules from the fibrils such that the soluble insulin concentration rose from negligible levels to ≈ 0.2 mg/ml (see Fig. 1). Hence, this experiment was performed to test whether this equilibrium could be established by fibril growth under these conditions, and not just fibril dissociation. Soluble insulin incubated at pH 9.5 in the absence of any fibrils was shown to remain completely soluble over a 400 h period (Fig. S4,

red circles). However, in the presence of 5% w/w preformed fibrils, the soluble insulin concentration decreased within this time period to 0.21 mg/ml (*open squares*), demonstrating that insulin fibril growth can occur slowly under alkaline conditions, and that an equilibrium between insulin fibrils and monomers is established by a balance of fibril elongation and dissociation.

A mathematical model to describe the observed dependence of dissociation rates on the pH

In order to examine the observed dependence of these fibril dissociation rates on hydrogen ion concentration, we modified a mathematical model, which has previously been used to rationalize the pH dependence of protein denaturation rates (6). Here, we assume that there is an equilibrium ($P^{-n} \rightleftharpoons P^{-(n+1)} + H^+$) within the fibrils between two states of the constituent protein molecules, P^{-n} and $P^{-(n+1)}$, with charges $-n$ and $-(n+1)$. Thus the equation for the relative concentrations of the two species is: $[P^{-(n+1)}] = \frac{K_0[P^{-n}]}{[H^+]}$ where K_0 is the equilibrium constant between the two species. Assuming that the more highly charged species can (irreversibly) dissociate far more readily than its less highly charged counterpart to yield the monomeric form, M, then the rate of monomer release is $d[M]/dt = k_1[P^{-(n+1)}]$ where k_1 is the rate constant for the process. Although this process is almost certainly not fully irreversible, it is reasonable to model the early stages in this way since the reaction will be significantly far from equilibrium. The rate constant k for formation of monomer must then be: $k = d[M]/dt\ \{[P^{-n}] + [P^{-(n+1)}]\}^{-1} = k_1K_0[P^{-n}]/[H^+]\ \{[P^{-n}] + K_0[P^{-n}]/[H^+]\}^{-1}$. In the region where $[H^+] \gg K_0$ (below the pK_a of the transition), we can simplify the previous expression to yield $k = k_1K_0/[H^+]$. This type of argument, therefore, predicts a linear dependence of $\log k$ on the pH as in Equation 3.

$$\log k = \log k_1 + \log K_0 - \log [H^+] \tag{3}$$

with gradient unity, and intercept $\log(k_1K_0)$.

We compare these theoretical considerations with the experimental data shown in Fig. 5*B*. As the plots of log k against pH have gradients that approach unity, our data strongly indicate that the kinetics of insulin fibril dissociation are regulated by the charge state of a single amino acid residue in the sequence. As the analysis stipulates that the pK_a of this residue must be higher than the pH range for which log k displays a linear dependence on pH, the pK_a of the amino acid must be above 12.0. This suggests a critical role for the unique arginine residue (pK_a 12.5) in the insulin sequence located at position B22.

References

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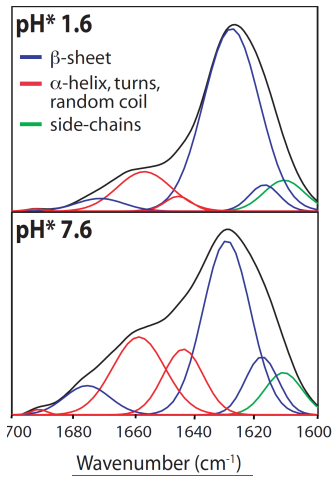

	pH*1.6		pH* 7.6	
	Peak (cm^{-1})	Area (%)	Peak (cm^{-1})	Area (%)
β-structure	1617	5.2	1618	9.6
	1628	66.1	1630	41.6
	1671	4.0	1675	6.7
Side-chains	1611	8.2	1611	7.9
Random coil	1646	2.6	1644	13.3
α-helix	1657	13.8	1658	20.6
Turns	1692	0.3	1691	0.4

Figure S1.

Secondary Structure of Insulin Fibrils. The amide I′ region of the FTIR spectra of insulin fibrils at pH* 1.6 and pH* 7.6 was deconvoluted; the coloured lines show the curve-fitting components. The line of best fits overlay the raw data. Resulting secondary structure composition is given in the table.

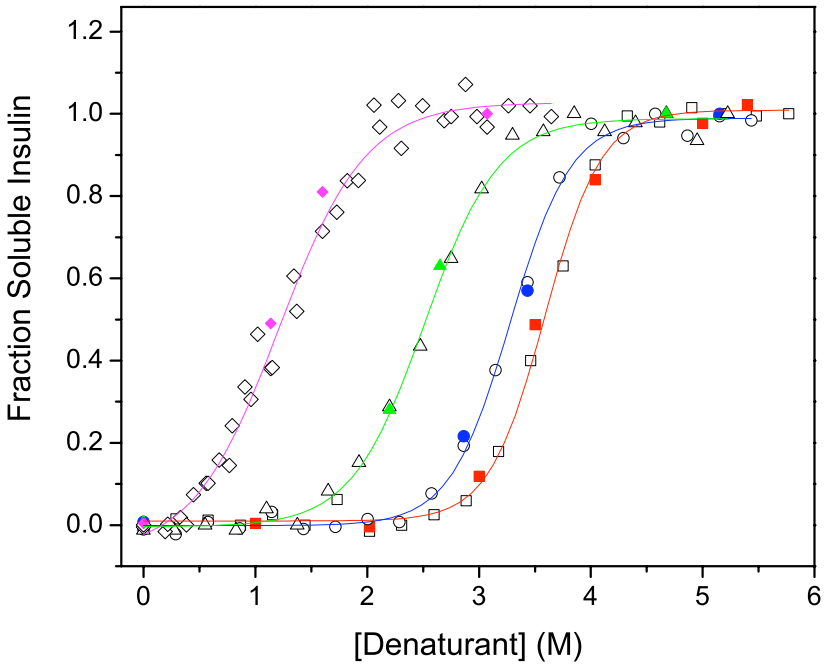

Figure S2.

Equilibration of the Fibril Dissociation Reaction in GdnSCN. Insulin fibrils were incubated in various concentrations of GdnSCN at pH 2.0 (*squares*), 4.0 (*circles*), 6.0 (*triangles*) and 8.0 (*diamonds*) and the fraction of soluble insulin determined after 24 h (*solid symbols*) and 48 h (*open symbols*). The lines are included to guide the eye.

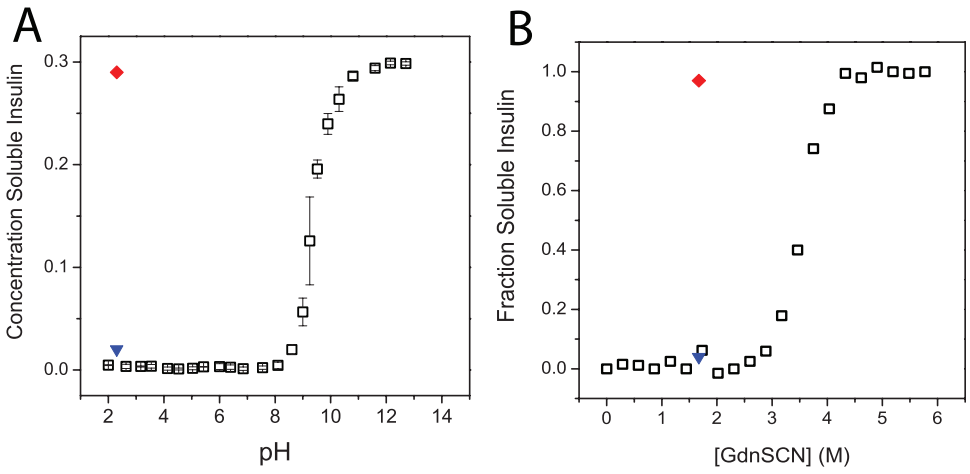

Figure S3.

Reversibility of the Fibril Dissociation Reaction Under Favorable Growth Conditions. Fibrils were dissociated by incubation at pH 11.0 or in the presence of 5 M GdnSCN at pH 2.0. These samples were then adjusted to more favourable solution conditions for fibril growth; the pH 11.0 sample was adjusted to pH 2.3 (A), and the GdnSCN sample was diluted such that the denaturant concentration was reduced to 1.7 M (B). The soluble concentration of insulin in each sample was determined at 0 h (*red diamonds*) and 48 h (*blue inverted triangles*). Complete datasets for the pH mediated and GdnSCN mediated dissociation of fibrils is shown for ease of interpretation (*open black squares*).

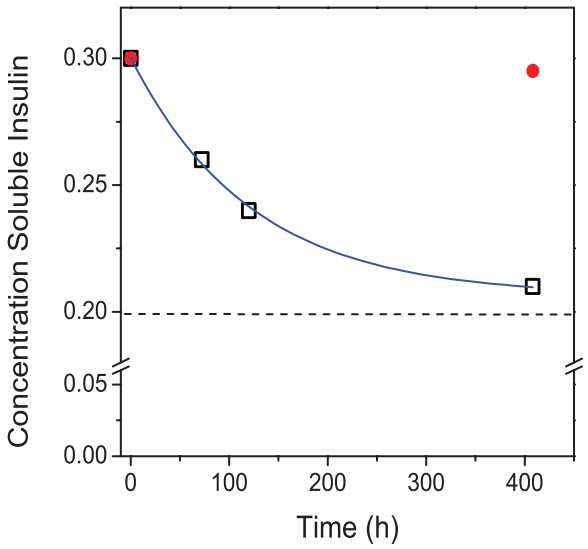

Figure S4.

Reversibility of Fibril Dissociation Reaction Under Unfavorable Growth Conditions. Solutions of soluble insulin at pH 9.5 and a protein concentration of 0.3 mg/ml were prepared with (*open black squares*) or without (*solid red circles*) the addition of 5% w/w preformed insulin fibrils (formed at pH 2.0). Solutions were incubated at room temperature and the soluble insulin concentration determined over a 400 h time period. The dashed line represents the concentration of soluble insulin liberated when a solution containing only fibrillar insulin at a final protein concentration of 0.3 mg/ml is adjusted to pH 9.4.