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NMR triple-quantum filtered relaxation analysis of ^{17}O-water in insulin solutions: an insight into the aggregation of insulin and the properties of its bound water

Allan M. Torres, Stuart M. Grieve, Philip W. Kuchel *

Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006, Australia

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Abstract

Transverse triple-quantum filtered NMR spectroscopy (TTQF) of ^{17}O-water was used to study the properties of water in insulin solutions at different Zn^{2+} concentrations and pH values. It was established that strongly bound water molecules are already present in Zn-free insulin. On the assumption that the effective correlation time of a strongly bound water molecule, τ_{sb}, is 10 ns, the apparent number of strongly bound water molecules was $\sim$3 to 4 per insulin monomer. Addition of Zn^{2+} equivalent to $\sim$2 g-atoms per hexamer did not produce substantial increases in the overall ^{17}O-water TTQF signal intensity and apparent fraction of bound water. The dramatic enhancement of the TTQF signals observed for samples with a Zn^{2+}/hexamer ratio greater than $\sim$2:1 could be attributed to the increase in correlation time of the strongly bound water, due to the formation of higher-order oligomers of the protein. © 1998 Elsevier Science B.V.

Keywords: NMR; ^{17}O-water; Bound water; Insulin; Hydration; Protein–water interaction; Triple-quantum-filtered relaxation analysis; Insulin aggregation

1. Introduction

The transverse triple-quantum filtered NMR experiment (TTQF) carried out on ^{17}O-water has been shown to be useful for determining the amount of bound water in biomolecules [1–3]. An important outcome of this experiment is that the amplitude of the observed TTQF ^{17}O-water signal in a given system of biomolecules is related to the number of strongly bound (sb) water molecules and/or to the magnitude of its correlation time, τ_{sb}, in the solution. Unlike relaxation–dispersion experiments, TTQF analysis of ^{17}O-water is easy to perform since it does not require several NMR instruments, or field cycling studies, that are necessary to cover a wide range of magnetic field strengths. However, the major shortcoming is that the population-size, and the correlation time, of the strongly bound water are, in effect, not statistically independent so one of the parameters must be known in order to determine the other. The correlation time of the strongly bound water in the protein solution is, therefore, usually taken as being known [3] and this was done in the present work.

* Corresponding author. Tel.: +61-2-93513709; fax: +61-2-93514726.

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A recent study has shown that the existence of calcium-coordinated water molecules in calbindin can be determined by NMR relaxation–dispersion experiments (NMRD) of ^{17}O-water [4]. It was shown that the apo-state of calbindin produces weak ^{17}O dispersion, corresponding to the presence of at least one strongly bound water molecule, while the calcium-loaded state gives relatively strong dispersion, indicating the presence of two long-residence calcium-coordinated water molecules. Therefore, it is seemed worthwhile to determine if the TTQF technique could also be applied to observe metal-coordinated water molecules in solutions of other proteins.

A good protein system to test the effectiveness of the TTQF technique is insulin. The structure of this important hormone has been comprehensively studied by various methods such as X-ray diffraction [5–7] and NMR spectroscopy [8–12]. X-ray studies on porcine 2-Zn-insulin hexamer crystals revealed water molecules tightly coordinated to the zinc [5–7] and we reasoned that these molecules could, in principle, be detected by TTQF or NMRD experiments. The hexamer is torus-shaped and the two Zn^{2+} ions are located in the central cavity separated by $\sim$1.7 nm [6,12]. Each of the Zn^{2+} ions is coordinated to three imidazolyl nitrogens of histidine residues, and three water molecules. Therefore, it is expected that at least six water molecules would be tightly bound to the hexamer. Apart from these findings, numerous protein-associated water molecules, which require further characterisation, have also been reported on the basis of X-ray studies of different crystalline forms of insulin [5,13,14].

In aqueous solution, Zn-free insulin exists as a mixture of oligomeric species consisting mostly of monomers, dimers, tetramers, and hexamers [15–21]. On the assumption that the oligomers undergo fast exchange of monomeric units, the observable NMR properties (e.g., signal amplitude and relaxation rate constant) will therefore be the weighted-average attribute of the different oligomeric states, present in the solution. The degree of association of Zn-free insulin is known to be highly dependent on pH, and ionic strength [22–25]. Self-association of insulin is greatest at pH 7.0 and, under this condition, addition of Zn^{2+} to a concentration slightly greater than 2 g-atoms per hexamer, can cause precipitation of the protein [23]. Since the TTQF signal intensity is highly dependent on the correlation time of the bound water, which is basically dependent on the protein's effective correlation time or aggregation size, it was considered that it may be possible to use the TTQF technique to probe the aggregation state of insulin; in the presence of various amounts of Zn^{2+}, and at different pH values.

Several insulin solutions with different Zn^{2+} concentrations were employed to determine the effect of Zn^{2+} ions on the relaxation properties of ^{17}O-water, and ultimately relate these results to the structure and dynamics of the bound water molecules in insulin.

2. Materials and methods

Porcine insulin (Nordisk Gentofte) was a gift from Wellcome Australia, N.S.W. Bovine insulin was purchased from Sigma (St. Louis, MO, USA), and 'heavy' water (21.5 at.% ^{17}O, 22.3 at.% ^{18}O) was obtained from Isotec (Miamisburg, OH, USA). Experiments were performed on both porcine and bovine insulin solutions, which consistently gave similar results; in view of our more extensive studies on porcine insulin, only results relating to it are presented here.

Insulin was dissolved by adding 1 ml of deionised water to 70 mg of insulin crystals, raising the pH to 10.5 using 0.1–1.0 M NaOH, and finally adjusting the pH to $\sim$8.5 with 0.1–1.0 M HCl. The method of removal of Zn^{2+} was based on that described previously [26]: specifically, 1 ml of the insulin solution was diluted in 20 ml of 0.05 M Tris–HCl pH 8.0. This was passed through a Chelex 100 column, and then washed and concentrated by ultrafiltration using a YM2 Amicon (Beverly, MA, USA) membrane. D_2O, which is known to contain a higher content of ^{17}O-water than ordinary 'reverse osmosis' water [1], was used in all washings so that the final solution of about 1.5 ml contained only traces of ^1H_2O and buffer. Further enrichment of ^{17}O-water spins was performed by adding 10 μl of the 'heavy' water.

Protein concentrations were determined spectrophotometrically at a wave length of 276 nm, using the fact that a 1 g ml^{-1} insulin solution has an absorbance of 1.05 cm^{-1}. The removal of Zn^{2+} in the bovine insulin sample was confirmed by atomic

absorption spectrophotometry using a Varian SpectrAA-20 Plus spectrometer (Varian Australia, Mulgrave, Vic., Australia). The pH of the sample was adjusted by adding small volumes of dilute NaOH or HCl.

All ^{17}O NMR experiments were performed on a Bruker AMX 400 wide-bore spectrometer operating at 54.24 MHz, and 298 K. Due to limited amount of sample, insulin solutions were dispensed into Wilmad 10-mm (o.d.) microcell tubes with sample capacity of 730 μl. In a typical experiment, the 90° pulse duration was 20 μs; spectral width 10 kHz; acquisition time 51 ms; effective relaxation time 52 ms; and acquired FID signals consisted of 1 k complex points. Except for the T_2 analysis, in which no window function was employed, all FID were processed with an exponential line broadening factor of 50 Hz.

Longitudinal relaxation rate constants were calculated from the data obtained using the regular inversion recovery experiment which employed a 90°_x–180°_y–90°_x composite inversion pulse. Transverse relaxation rate constants were approximated from the linewidth at half the maximum peak amplitude, obtained in a conventional pulse-and-acquire experiment. Transverse triple-quantum filtered experiments were performed according to the sequence described by Baguet et al. [1] and Chung and Wimperis [27], which is 90°-τ_e/2–180°-τ_e/2–70.5-τ_m–90°-acq(t), with appropriate phase cycles. The data were acquired as a pseudo 2D-experiment in which the echo delay, τ_e, was incremented for each FID consisting of 2 k transients while the mixing delay, τ_m, was fixed at 4 μs. The total time for each TTQF experiment was $\sim$1.5 h.

Estimation of the apparent fraction of bound water, p_{sb}, was carried out by a restricted non-linear least-squares fit of the relevant expression to the observed TTQF signal profiles, as described previously [3]. In this procedure, a trial 3×3 ^{17}O-water relaxation matrix is first set-up using the master equation:

$$\mathbf{R}^{(n)}_{\text{system}} = \sum_i p_i \mathbf{R}^{(n)}_i \qquad (1)$$

where $\mathbf{R}$ is the Redfield relaxation matrix which contains terms involving quadrupolar coupling constants and spectral density functions, J, that are a function of the correlation time τ_c; n is the coherence order whose value is 1 for the transverse TQF signal; $\mathbf{R}^{(n)}_{\text{system}}$ is the resulting relaxation matrix; $\mathbf{R}^{(n)}_i$ is the corresponding relaxation matrix for state i, in the absence of chemical exchange; and p_i is the fraction of ^{17}O-water in state i. The elements of the relaxation matrix $\mathbf{R}$ are explicitly defined in [27]. A pseudo two-state fast-exchange model consisting of strongly bound (sb) and free water ('free') was assumed so that Eq. (1) can be expressed as:

$$\mathbf{R}^{(1)}_{\text{system}} = p_{\text{sb}} \mathbf{R}^{(1)}_{\text{sb}} + (1 - p_{\text{sb}}) R^{(1)}_{\text{free}}, \qquad (2)$$

The relaxation matrix $\mathbf{R}^{(1)}_{\text{free}}$, was constructed on the basis of the value of the longitudinal relaxation R_{free}, obtained from an inversion recovery experiment. It can be written as:

$$R^{1\text{free}} = \begin{bmatrix} R_{\text{free}} & 0 & 0 \\ 0 & \frac{33}{8} R_{\text{free}} & 0 \\ 0 & 0 & \frac{15}{8} R_{\text{free}} \end{bmatrix} \qquad (3)$$

while $\mathbf{R}^{(1)}_{\text{sb}}$ values were obtained by assuming $\tau_{\text{sb}} = 10$ ns; the Larmor frequency $(\omega_0/2\pi)$ was 54.24 MHz and the 'quadrupolar constant' $(e^2qQ/h)(1 + \eta^2/3)^{1/2}$ for strongly bound water is 7.6 MHz. By adopting a trial value for p_{sb}, the two terms on the right hand side were then added to obtain finally the total relaxation matrix $\mathbf{R}^{(1)}_{\text{system}}$, which was then diagonalized, as represented by the equation:

$$\mathbf{R}^{(1)}_{\text{system}} = \mathbf{V}^{(1)-1} \mathbf{R}^{(1)}_{\text{diag}} \mathbf{V}^{(1)} \qquad (4)$$

where $\mathbf{V}^{(1)}$ is the eigenvector matrix and $\mathbf{R}^{(1)}_{\text{diag}}$ is the eigenvalue matrix of diagonal elements $R^{(1)}_q$. The TTQF coherence decay (relaxation) function is now constructed using the equation:

$$f^{(1)}_{31}(\tau_e) = \sum_{q=1}^{5} \mathbf{V}^{(1)}_{q^3} \mathbf{V}^{(1)}_{qi} \exp\left(R^{(1)}_q \tau_e\right) \qquad (5)$$

It was the value of this function that was compared with the experimental TTQF signal intensity. Iterative fitting was performed by adjusting p_{sb} and a scaling factor A so that the sum-of-squares deviation between values calculated using the signal decay function $f^{(1)}_{31}(\tau_e)$ and the experimentally acquired signal was minimised.

3. Results

3.1. Zn^{2+} titration of insulin solutions

3.1.1. Longitudinal and transverse relaxation

Fig. 1 shows plots of the longitudinal (R_1) and transverse (R_2) relaxation rate constants of ^{17}O-water in an 8.9-mM solution of insulin, as a function of Zn^{2+} concentration, at pH 8.13, and 298 K. Clearly, in every case R_2 was significantly greater than the corresponding R_1 value. Addition of Zn^{2+} to the insulin solution resulted in a general increase in both the longitudinal and transverse relaxation rate constants of ^{17}O-water, although the increase in R_1 was virtually insignificant. The dependence of R_2 on the Zn^{2+} concentration was non-linear and had a larger slope at higher Zn^{2+} concentrations.

3.1.2. Transverse triple-quantum filtered relaxations

Fig. 2 shows plots of the ^{17}O-water TTQF signal intensities in a solution of insulin, as a function of the pulse-sequence delay, τ_e, and with various Zn^{2+} concentrations. It is readily apparent that even the Zn-free solution yielded a pronounced TTQF signal, thus indicating the presence of tightly bound water in this 'control' sample. The TTQF signal intensity increased with an increase in the concentration of Zn^{2+}. The rise in TTQF signal intensity was gradual for a Zn^{2+} concentration up to 1.8 g-atoms per hexamer, but it became more dramatic at higher Zn^{2+} concentrations.

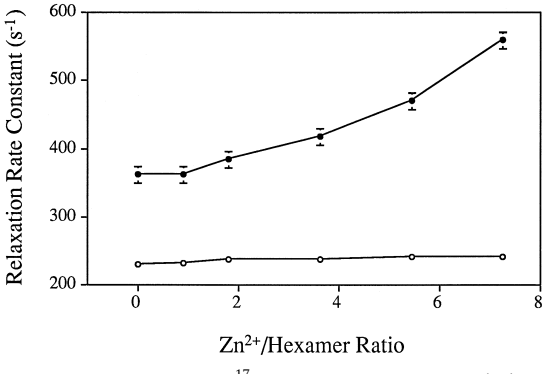

Fig. 1. The graph of the ^{17}O-water longitudinal R_1 (○) and transverse relaxation R_2 (●) rate constants, in an 8.9-mM porcine insulin solution, as a function of the ratio of the number of Zn^{2+} ions per hexamer, at pH 8.13, and 298 K. The error bars for the R_1 values were $\sim \pm 0.3\ s^{-1}$, so they lie within the confines of the symbols.

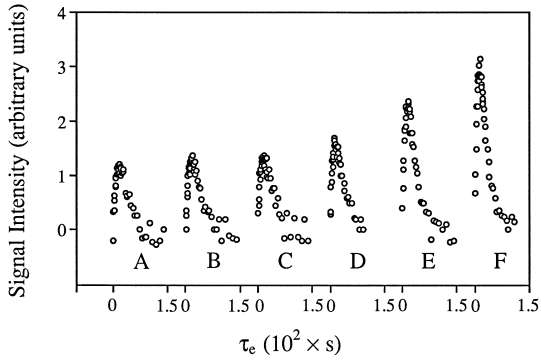

Fig. 2. The ^{17}O-water TTQF signal intensities from an 8.9-mM insulin solution with various Zn^{2+} concentrations, plotted as function of the pulse-sequence delay, τ_e. The ratio of the number of Zn^{2+} ions per hexamer was: 0.0:1.0 (A); 0.9:1.0 (B); 1.8:1.0 (C); 3.6:1.0 (D); 5.4:1.0 (E); and 7.3:1.0 (F). The signals were plotted as absolute intensities.

The apparent fraction of strongly bound water, p_{sb}, was calculated for each situation by non-linear least-square fitting of the TTQF signal–relaxation profiles [3], using the experimentally obtained R_1, and assuming an effective correlation time (τ_{sb}) of 10 ns. The adopted τ_{sb} value was based on the NMRD study conducted by Halle et al. [28] which showed that bound water has a correlation time, referring to the slow motion, of the order of 10 ns. Fig. 3 presents the apparent fraction of strongly bound water, p_{sb}, in an 8.9-mM insulin solution with different Zn^{2+} concentrations. The aggregation state of insulin in solution is not homogenous, but is a mixture of different oligomers, so the effective correlation time, τ_{sb}, will differ significantly in the different aggregates. Thus, the p_{sb} values calculated are unlikely to be rigorous estimates of the fractions of strongly bound water in solution. Instead, the estimated p_{sb} values reflect either variations in the fraction of strongly bound water or changes in the effective correlation time, τ_{sb}, or both. As shown in Fig. 3, there was no significant difference in p_{sb} values for Zn-free insulin, and those complexes with a Zn^{2+}/hexamer ratio of 0.9:1.0 and 1.8:1.0. The increase in p_{sb} was more substantial when the Zn^{2+}/hexamer ratio was increased above 1.8:1.0;

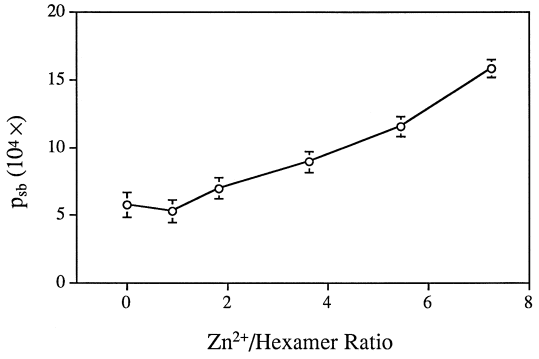

Fig. 3. The calculated apparent fraction of strongly bound water, p_{sb}, in an 8.9-mM insulin solution, plotted as a function of the Zn^{2+}/hexamer ratio. Values for p_{sb} were obtained by non-linear least-squares fitting to the observed TTQF signal profile, assuming an effective correlation time, τ_{sb}, of 10 ns.

this trend was similar to that observed for the variation in TTQF signals in Fig. 2.

3.2. pH titration of insulin solution

Fig. 4 shows the TTQF signal profiles for a 12.6-mM solution of Zn-free insulin at different pH values. The TTQF signals were readily detected in all cases, thus suggesting the presence of strongly bound water at all the pH values studied. TTQF signals were largest for pH 3.36 and smallest for pH 10.57. Signal amplitudes at pH 2.09 were considerably smaller than those at pH 3.36, but were slightly larger than those at pH 8.12. Note that although no precipitate was observed at the start of the TTQF experiment at pH 8.12, a considerable amount of solid was formed after 12 h. This precipitation was due to the (slow) aggregation caused by the high insulin concentration and increased salt concentration that was the result of the pH adjustments. An increase in ionic strength can lead to aggregation of some protein solutions, because the ions reduce electrostatic repulsion between protein molecules, thus enabling hydrophobic interactions to be more pronounced.

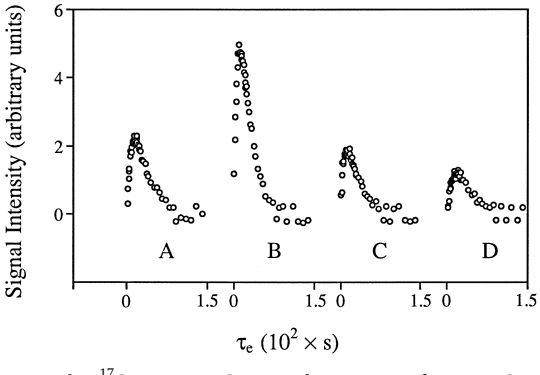

Fig. 4. The ^{17}O-water TTQF signal intensities for a 12.6-mM insulin solution with pH values of 2.09 (A), 3.36 (B), 8.12 (C), and 10.57 (D), plotted as a function of the pulse-sequence delay τ_e. The signals were plotted as absolute intensities.

4. Discussion

4.1. Zn^{2+} titration of insulin solutions

When interpreting the results of the Zn^{2+}-titration study, it is useful to consider first the aggregation and zinc-binding properties of the insulin samples. On the basis of the association constants reported for pH 8.0 [21], the expected composition of the different oligomers in the 8.9-mM Zn-free insulin solution was 1% monomer, 64% dimer, 17% tetramer, and 18% hexamer. It is anticipated that Zn^{2+} addition, corresponding to a Zn^{2+}/hexamer ratio of 2:1 would completely transform the lower order oligomers in the solution into hexamers. Insulin at pH 8.0 can bind a total of 18 g-atoms of Zn^{2+}/hexamer [21]. The stronger binding sites have a stoichiometric binding of about 6 Zn g-atoms per hexamer, while the weaker binding sites have 12 g-atoms per hexamer. Thus, in most experiments performed in the Zn^{2+} titration experiments, it can be considered that all Zn^{2+} ions were tightly associated with the insulin.

The variation of the apparent longitudinal, and transverse, relaxation rate constants (each a single value) characterising the relaxation of the total ^{17}O-water signal in the insulin solutions, with various Zn^{2+} concentrations, provided initial evidence of fast exchange of the bulk water with a small population of tightly bound molecules (Fig. 1). Note also that the values of the R_2 rate constants, given in Fig. 1, included an extra contribution from magnetic field inhomogeneity; however, this is expected to be small compared to the contribution from quadrupolar re-

laxation. Moreover, care must be taken in interpreting R_2 since in the non-fast-motional narrowing regime, the relaxation is non-exponential and only apparently described by a single value of R_2. This is in contrast to the longitudinal relaxation which is very well approximated by a single exponential with a rate constant of R_1, regardless of the value of the effective correlation time of the protein. Hence, the R_2 values should only be taken as approximations to the real values. The increase in R_1 and R_2 values with increasing concentration of Zn^{2+} (Fig. 1) indicated that the Zn^{2+} caused changes in the structure and dynamics of water in the solution. Since the increase in R_2 was much larger than that of R_1, the R_2/R_1 ratio increased; this suggests a shift in relaxation behaviour of water from a 'liquid'-like to a more 'solid'-like state as the amount of bound Zn^{2+} increased. Based on the R_1 and R_2 data alone, it would not be possible to describe, accurately the changes in the amount and correlation time of the weakly and strongly bound water. The dramatic increase in R_2 vs. R_1 can, perhaps, be attributed to increases in the effective correlation times of both the strongly and weakly bound water molecules, which occurs with insulin aggregation; the expression for R_2 includes a spectral density term J_0 which is directly proportional to the correlation time. Thus, these relaxation data were consistent with the observation of small amounts of gel-like precipitate in insulin solutions with Zn^{2+}/hexamer ratios greater than 5.4:1.0; the main bulk of such precipitates had been separated from the solutions before NMR experiments were performed.

In contrast to the longitudinal and transverse relaxation data, the TTQF signal intensity profiles shown in Fig. 2 provided a clear impression of the properties of the strongly bound water in the insulin solution. The fact that the Zn-free insulin solution produced significant TTQF signals indicates that tightly bound water molecules were already present in the insulin solution at 8.9 mM and pH 8.13. Based on the assumption that the effective correlation time, τ_{sb}, is 10 ns, the apparent fraction of strongly bound water, p_{sb}, was estimated to be 0.00057 ± 0.00009, corresponding to ~ 3 to 4 water molecules per insulin monomer. The observed increase in TTQF signal intensity with increasing amount of Zn^{2+} indicates that there was an increase in the amount, and/or correlation time, τ_{sb}, of the strongly bound water; thus, the longitudinal and transverse relaxation behaviour agrees with the data presented in Fig. 1.

As mentioned earlier, increasing the amount of bound Zn^{2+} to 2 g-atoms per hexamer leads to total conversion of lower order aggregates into hexamers. This aggregation process would result in an increase in the 'effective' correlation time of the bound water or insulin. Thus, part of the increase in R_2, and overall TTQF signal intensities upon increasing the Zn^{2+} per hexamer ratio to 1.8:1.0, can be attributed directly to the formation of hexamers. It was predicted that a dramatic increase in TTQF signal might result from the formation of hexamers, due to the binding of the two Zn^{2+} ions, which in turn are known to coordinate six water molecules. However, as is clearly shown in Figs. 2 and 3, the predicted large increase in the signal did not occur and the increase in the apparent bound fraction, p_{sb}, was, in fact, insignificant. The presence of several oligomeric species made it difficult to quantify the changes in p_{sb}, that occurred upon increasing the Zn^{2+}/hexamer ratio up to 1.8:1.0. Nevertheless, it can be inferred from the results in Figs. 2 and 3 that lower-order aggregates, particularly dimers and tetramers, already contained many strongly bound water molecules whose relaxation properties were not substantially different from those of the strongly bound water in the hexamers. It is also possible that the formation of hexamers does lead to the tight binding of six water molecules in the core of the hexamer; but these water molecules could be in slow exchange with the solvent, so that their presence would not be detected in the relaxation analysis. This notion of 'unobservable' slowly-exchanging bound-water raises the possibility that some water molecules could also be trapped between oligomeric interfaces as higher order aggregates are formed.

Equilibrium sedimentation studies on the aggregation of insulin have shown that higher-order oligomers, larger than hexamers, can be created when appropriate concentrations of Zn^{2+} are added to insulin solutions [22,25]. In a study at pH 7.3 [22], it was found that the sedimentation coefficient of insulin increased linearly when the Zn^{2+}/hexamer ratio was increased above ~ 2.4:1.0. In a previous related study conducted at pH 8, increasing the

Zn^{2+}/hexamer ratio led to the observation of a 72,000 Da (dodecamer) species; and when the ratio was increased to 6:1, oligomers with molecular mass between 200,000 and 300,000 Da were observed [25]. In yet another study conducted at pH 7.0, insulin formed insoluble aggregates, larger than hexamers when more than 2 g-atoms Zn^{2+} were bound per hexamer [23]. Although these higher order aggregates were insoluble at neutral pH, they may have disaggregated at other pH values, as was shown in the present work. Specifically, at pH 8.13, gel-like precipitates were observed only after the Zn/hexamer ratio reached 5.4:1.0. Although these precipitates were separated from the solutions prior to the NMR measurements, it is still likely that the solutions contained a large fraction of higher order aggregates.

It is clear that aggregates larger than hexamers were formed in our experimental solutions beyond a 2.0:1.0 Zn^{2+}/hexamer ratio. In interpreting TTQF results beyond this ratio, care has to be taken since the effective correlation time of strongly bound water, τ_{sb}, will probably increase significantly with an increase in the rotational correlation time of the protein, τ_r, caused by aggregation. This is assuming that the residence times τ_{res} of these bound water molecules are longer than τ_r. Provided that these large aggregates of insulin stay dissolved in the solution, the increase in τ_{sb} would lead to an increase in the TTQF signal, and substantial narrowing of the overall signal–relaxation rate profile. As shown in Fig. 2D–F, this appeared to be the case, although an increase in the actual p_{sb} without any significant change in τ_{sb} could equally well explain the same trend. The latter explanation is however improbable due to the occurrence of aggregation under high Zn^{2+} concentrations.

Unlike in previous cases where the Zn^{2+}/hexamer ratio was less than 2.0:1.0, it would be difficult to speculate on the effective correlation time, τ_{sb}, for each situation above this threshold ratio, since the apparent molecular mass of insulin tends to increase significantly with the concentration of Zn^{2+}. For simplicity, we used an apparent τ_{sb} value of 10 ns in all calculations. Any substantial change in actual τ_{sb} value would be reflected in the estimated p_{sb} values since these two parameters are, effectively, not statistically independent in the TTQF experiments [3]. The fact that no significant change in p_{sb} was obtained upon increasing the Zn^{2+}/hexamer ratio to 1.8:1.0 implies that no substantial change in τ_{sb} occurred within this range. The dramatic enhancement of the TTQF signals observed when the Zn/hexamer ratio exceeded 1.8:1.0 led to a significant increase in the apparent bound fraction, p_{sb} (see Fig. 3). This effect may be attributed, primarily, to the increase in effective correlation time τ_{sb}, due to the formation of larger aggregates, when additional Zn^{2+} became bound to the insulin.

Although the increase in the correlation time of bound water, due to aggregation, appeared to satisfactorily explain the substantial rise in TTQF signal, we cannot discount the possibility that there were substantial contributions to the TTQF signal from an increase in the number of strongly bound water molecules, when the Zn^{2+}/hexamer ratio was increased. This is plausible because additional Zn^{2+} ions can coordinate water molecules; thus as Zn^{2+} bound to insulin additional water molecules could have been trapped between the oligomeric species in the larger aggregates. NMRD experiments on the insulin solution with different Zn^{2+} concentration would be useful to address this suggestion.

4.2. pH titration of insulin solutions

The results presented thus far showed clearly that solutions containing larger aggregates of insulin yielded stronger TTQF signals. Since insulin aggregation is known to be highly dependent on pH, the relative variation of the TTQF signal amplitudes with pH provided a useful insight into the aggregation state(s) of Zn-free insulin. The veracity of this idea was tested with lysozyme whose aggregation size in solution is known to increase with increasing pH values [29]. There was a substantial increase in the TTQF signal intensity with pH, which occurred with an increase in cloudiness of the solution.

Thus, the data in Fig. 4 suggest that lower-order aggregates of insulin are more favoured at pH 10.57, than at pH 2.09, 3.36, and 8.12, since the TTQF signal at this pH value was the smallest. It may be inferred also that the extent and nature of the aggregation at pH 2.09 was similar to that at pH 8.12, since maximum TTQF signal intensity of the two were very similar. These results are consistent to

those obtained with sedimentation equilibrium analysis: a study conducted by Mark et al. [16] found that aggregation of Zn-free insulin is greatest at pH 7.0 and increases in the order pH 10.0 < 2.0 < 7.0. In a related sedimentation study, Goldman and Carpenter [21] found that the association pattern at pH 8.0 was similar to that at pH 2.0.

Finally, it should be noted that the precipitation which occurred during an NMR experiment at pH 8.12 most probably caused the TTQF signal to decrease because this aggregation process leads to a smaller amount of protein dissolved in solution. However, since this aggregation process was found to occur slowly over 12 h, and the TTQF experiment was completed by 1.5 h, the effect of aggregation on the maximum signal intensity in the TTQF experiment was expected to be minimal.

The above results clearly showed that TTQF analysis of ^{17}O-water could be used to probe the aggregation state of insulin. The technique should be applicable to other proteins provided that strongly bound water molecules are present and the residence time(s), τ_{res}, of these are longer than the rotational correlation time of the protein aggregates, τ_r, so that τ_{sb} is effectively equal to τ_r.

5. Conclusions

At the beginning of this study, it was anticipated that strong ^{17}O-water TTQF signals would be obtained from zinc-coordinated water molecules in the insulin hexamer. However, this was not the case since the addition of Zn^{2+} equivalent to ~2:1 Zn^{2+}/hexamer did not lead to a significant enhancement of the TTQF signals. Intense signals from Zn-free insulin clearly suggested that the presence of strongly bound water molecules is independent of Zn^{2+} binding. The structural and dynamical changes in water molecules, that occur as insulin binds Zn^{2+}, are presumably affected by the presence of several oligomeric species of insulin. Solutions with larger aggregates of insulin brought about by changing pH provided stronger ^{17}O-water TTQF signals; thus changes in the aggregation state of insulin could be inferred from the TTQF spectra and the subsequent analysis.

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References

[1] E. Baguet, B.E. Chapman, A.M. Torres, P.W. Kuchel, J. Magn. Reson. Ser. B. 111 (1996) 1.
[2] C.W. Flesche, M.L.H. Gruwel, A. Deussen, J. Schrader, Biochim. Biophys. Acta 1244 (1995) 253.
[3] A.M. Torres, S.M. Grieve, B.E. Chapman, P.W. Kuchel, Biophys. Chem. 67 (1997) 187.
[4] V.P. Denisov, B. Halle, J. Am. Chem. Soc. 117 (1995) 8456.
[5] E.N. Baker, T.L. Blundell, J.F. Cutfield, S.M. Cutfield, E.J. Dodson, G.G. Dodson, D.R. Crowfoot Hodgkin, R.E. Hubbard, N.W. Isaacs, C.D. Reynolds, K. Sakabe, N. Sakabe, N.M. Vijayan, Philos. Trans. R. Soc. London Ser. B 319 (1988) 369.
[6] T. Blundell, G. Dodson, D. Hodgkin, D. Mercola, Adv. Protein Chem. 26 (1972) 279.
[7] M.J. Adams, T.L. Blundell, E.J. Dodson, G.G. Dodson, M. Vijayan, E.N. Baker, M.M. Harding, D.C. Hodgkin, B. Rimmer, S. Sheat, Nature 224 (1969) 491.
[8] Q. Hua, M.A. Weiss, Biochemistry 30 (1991) 5505.
[9] E. Jacoby, Q.X. Hua, A.S. Stern, B.H. Frank, M.A. Weiss, J. Mol. Biol. 258 (1996) 136.
[10] J.H. Bradbury, V. Ramesh, G. Dodson, J. Mol. Biol. 150 (1981) 609.
[11] K.L. Williamson, R.J.P. Williams, Biochemistry 18 (1979) 5966.
[12] J.L. Sudmeier, S.J. Bell, M.C. Storm, M.F. Dunn, Science 212 (1981) 560.
[13] J. Badger, D.L.D. Caspar, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 622.
[14] J.M. Goodfellow, N. Thanki, J.M. Thornton, in: E. Westhof (Ed.), Hydration of Amino Acids in Protein Crystals (Water and Biological Macromolecules), Macmillan, London, 1993, p. 63.
[15] P.D. Jeffrey, J.H. Coates, Biochemistry 5 (1966) 489.
[16] A.E. Mark, L.W. Nichol, P.D. Jeffrey, Biophys. Chem. 27 (1987) 103.
[17] Y. Pocker, S.B. Biswas, Biochemistry 20 (1981) 4354.
[18] A.H. Pekar, B.H. Frank, Biochemistry 11 (1972) 4013.
[19] P.D. Jeffrey, B.K. Milthorpe, L.W. Nichol, Biochemistry 15 (1976) 4660.
[20] A.E. Mark, P.D. Jeffrey, Biol. Chem. Hoppe-Seyler 371 (1990) 1165.
[21] J. Goldman, F.H. Carpenter, Biochemistry 13 (1974) 4566.

[22] L.W. Cunningham, R.L. Fischer, C.S. Vestling, J. Am. Chem. Soc. 77 (1955) 5703.
[23] P.T. Grant, T.L. Coombs, B.H. Frank, Biochem. J. 126 (1972) 433.
[24] W. Kadima, M. Roy, R.W.-K. Lee, N.C. Kaarsholm, M.F. Dunn, J. Biol. Chem. 267 (1992) 8963.
[25] E. Fredericq, Arch. Biochem. Biophys. 65 (1956) 218.
[26] M.F. Dunn, S.E. Pattison, M.C. Storm, E. Quiel, Biochemistry 19 (1980) 718.
[27] C.-W. Chung, S. Wimperis, Mol. Phys. 76 (1992) 47.
[28] B. Halle, T. Andersson, S. Forsén, B. Lindman, J. Am. Chem. Soc. 103 (1981) 500.
[29] M.R. Bruzzesi, E. Chiancone, E. Antonini, Biochemistry 4 (1965) 1796.