The Molecular Chaperone α-Crystallin as an Excipient in an Insulin Formulation

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ABSTRACT

Purpose To investigate insulin fibrillation under accelerated stress conditions in the presence of a novel excipient, the molecular chaperone α-crystallin, in comparison with common excipients.

Methods To induce fibrillation, recombinant human insulin (0.58 mg ml\(^{-1}\)) formulations without excipient or with bovine α-crystallin (0.01–0.2 mg ml\(^{-1}\)), human serum albumin (1–5 mg ml\(^{-1}\)), sucrose (10–100 mg ml\(^{-1}\)) or polysorbate 80 (0.075–0.3 mg ml\(^{-1}\)) were subjected to stirring stress in a fluorescence well plate reader and formulation vials. Protein fibrillation was monitored by thioflavin T. The formulations were further characterized by size-exclusion chromatography, light obscuration, UV/Vis and circular dichroism spectroscopy.

Results In both methods, insulin formed thioflavin T-binding species, most likely fibrils. Addition of α-crystallin in the well plate assay greatly improved insulin’s resistance to fibrillation, measured as a 6-fold increase in fibrillation lag time for the lowest and 26-fold for the highest concentration used, whereas all other excipients showed only a marginal increase in lag time. The stabilizing effect of α-crystallin was shown by all characterization techniques used.

Conclusions The effect of α-crystallin on insulin’s physical stability outperforms that of commonly used excipients. α-Crystallin is proposed to bind specifically to pre-fibrillation species, thereby inhibiting fibrillation. This makes α-crystallin an interesting excipient for proteins with propensity to fibrillate.

KEY WORDS fibrillation · formulation design · insulin · protein excipients · α-crystallin

ABBREVIATIONS

bis-ANS 4,4′-Bis(1-anilinonaphthalene 8-sulfonate)
FUV-CD Far ultraviolet circular dichroism
HP-SEC High performance size-exclusion chromatography
HSA Human serum albumin
NUV-CD Near ultraviolet circular dichroism
ThT Thioflavin T

INTRODUCTION

Protein pharmaceuticals are normally administrated by injections because of their poor absorption through oral, mucosal or transdermal routes. Therapeutic proteins are formulated as a liquid or lyophilized product comprising a suitable buffer and a selection of excipients designed to maximize protein stability (1,2). Furthermore, most formulations have to be kept in cold storage, even when lyophilized, to prevent the degradation of the therapeutic protein which can occur by chemical pathways (deamidation, oxidation, etc.) or physical pathways (misfolding or unfolding), which both can lead to the formation of aggregates (reviewed in (1–4)). Aggregates compromise the safety and efficacy of the therapeutic protein and may increase its immunogenicity (5,6).

Due to the great diversity in protein sequence, structure and, consequently, routes of degradation, no single formulation strategy can be generally applied to all proteins. Instead, a suitable tailor-made formulation will have to be developed for each therapeutic protein. The search for novel excipients to stabilize protein formulations is, therefore, an ongoing process.

An important problem related to therapeutic formulations of primarily smaller proteins and peptides, such as
insulin, calcitonin, glucagon and glucagon-like peptides (7), is the formation of amyloid fibrils. Insulin is one of the best described amyloid systems, and its fibrillation is considered a nucleation-dependent process (8). The first step of the fibrillation process is the reversible dissociation of the insulin hexamer or tetramer to dimer or monomer. The dimer or monomer unfolds to a partially folded intermediate (9,10). The formation of this intermediate is promoted by temperature, agitation and the presence of air-water interface and surfaces (11,12). When a critical concentration of partially folded intermediate is reached, the formation of stable fibrillation nuclei, from which the mature fibrils grow, are possible (9). This accumulation of intermediates has very little apparent effect on the bulk formulation. The events leading to formation of the nuclei are generally referred to as the lag phase.

The formation of nuclei is followed by their extension into fully grown fibrils (exponential growth phase). The formation of the fibrillation nuclei is energetically unfavorable and suggested to be a stochastic event, where only a single or very small number of nuclei are formed (13,14). The dependency on a very limited number of events is suggested to account for the high variance in lag time generally observed in fibrillation studies (13,14). After the formation of the first stable fibril, secondary nucleation, where the formation of new nuclei is catalyzed by the already formed fibrils, is proposed to explain the rapid conversion of the bulk native protein to fibrils (13).

As prevention of protein fibrillation during production, transport and storage remains a challenge to the pharmaceutical industry, we have evaluated α-crystallin, a member of the small heat shock protein superfamily, as a novel anti-fibrillation excipient in a formulation of recombinant human insulin.

The novel excipient studied in this report, the chaperone-like protein α-crystallin, is a polydisperse oligomer native to the vertebrate eye lens. The oligomer consists of 15–50 of the homologous αA and αB-crystallin subunits in an estimated 3:1 ratio (15–17). As both subunits have an approximate molecular mass of 20 kDa, the polydisperse oligomer has a size range between 300 and 1,000 kDa (15). The physiological function of α-crystallin is to prevent protein aggregates (also called cataracts) from forming in the eye lens (18). This chaperone-like effect of α-crystallin has also been shown in a range of protein systems in vitro ((18,19) and reviewed in (20,21)). The proposed mechanism of the protective effect of α-crystallin is an interaction with the exposed hydrophobic surfaces of partially denatured or misfolded proteins (22,23), thereby preventing their self-association and irreversible aggregation. The chaperone-like function of α-crystallin is independent of chemical energy input and highly selective for destabilized proteins (24). α-crystallin activity is further enhanced above physiological temperature, which increases the capacity and affinity for binding its substrates (19,25). Several members of the small heat shock protein superfamily, including α-crystallin, are reported to be effective inhibitors of amyloid fibrillation (26,27).

The aim of this study was to test α-crystallin as a stabilizing excipient for recombinant human insulin, a therapeutic protein with a propensity to fibrillate. The stabilizing effect of α-crystallin was compared to that of human serum albumin (HSA), a protein excipient used commonly in several therapeutic protein formulations (28,29). HSA has been claimed to have some chaperone-like function (30). Furthermore, we also compared α-crystallin activity against the widely used excipients polysorbate 80 (commercially known as Tween® 80) and sucrose (28). The stabilizing effect of the excipients was tested in two insulin fibrillation assays, both applying stirring to stress the formulations, one in a fluorescence well plate and one in a formulation vial.

**MATERIALS AND METHODS**

**Materials**

Recombinant human insulin was kindly donated by Schering-Plough (Oss, the Netherlands). HSA and bovine α-crystallin were procured from Sigma-Aldrich (St. Louis, US) and used without further purification. Polysorbate 80 was purchased from Merck-Schuchardt (Hohenbrunn, Germany). All other chemicals were of analytical grade.

Insulin was dissolved in a minimal amount of 0.05 M hydrochloric acid, then diluted in a large excess of phosphate buffer (50 mM phosphate with 100 mM NaCl and adjusted to pH 7.4 with sodium hydroxide). HSA and α-crystallin were dissolved directly in the phosphate buffer. The concentrations were determined by UV absorption, using a molecular weight of 5,808 g·mol⁻¹ and ε_{276 nm} of 6,200 M⁻¹ cm⁻¹ for insulin (31) and a molecular weight of 66,500 g·mol⁻¹ and ε_{279 nm} of 35,300 M⁻¹ cm⁻¹ for HSA (32). For α-crystallin, a molecular weight of 19,852 g·mol⁻¹ and an ε_{280 nm} of 13,300 M⁻¹ cm⁻¹ for an average monomer was used, based on a 3:1 αA to αB-crystallin ratio as described in (33).

**Insulin Formulations**

The formulations were prepared as 0.58 mg ml⁻¹ insulin (0.1 mM) in phosphate buffer. The zinc content of the insulin formulation was approximately three zinc ions per hexamer. Three formulations were used in the vial assay (see below): an insulin formulation with no addition of excipient, a formulation containing 0.2 mg ml⁻¹ α-crystallin.
and one containing 2.5 mg ml\(^{-1}\) HSA. The insulin formulation used in the well plate assay (see below) either contained no excipients or was co-formulated with \(\alpha\)-crystallin (0.01–0.2 mg ml\(^{-1}\), HSA (1–5 mg ml\(^{-1}\)), sucrose (10–100 mg ml\(^{-1}\)) or polysorbate 80 (0.075–0.3 mg ml\(^{-1}\)).

**Vial Assay**

The insulin formulations (4 ml) were transferred into 6 ml Ø 20 mm glass type 1 vials with a Teflon-coated rubber stopper. The samples were stirred in upright position using a 12 mm Ø 4 mm Teflon-coated stirring bar on a multipoint magnet stirrer set at 750 rpm. The experiments were carried out at 5°C, 25°C and 45°C (±1°C). The stirring was ensured to be symmetrical so the formulation had no contact with the vial neck or stopper. Samples were taken out and analyzed using high performance size-exclusion chromatography (HP-SEC), fluorescence and UV spectroscopy, circular dichroism, and light obscuration.

**Well Plate Assay**

An accelerated stress study was set up in a FLUOstar OPTIMA (BMG, Offenburg, Germany) fluorescence plate reader. Two-hundred \(\mu\)L of the formulation with 20 \(\mu\)M thioflavin T (ThT) present was transferred into the wells of a polystyrene 96-well plate (Nunc, Rochester, US). A 3 mm glass bead (Whitehouse Scientific, Waverton, UK) was put in each well to ensure reproducible stirring, and the plate was covered using polyolefin sealing tape (Nunc, Rochester, US) to prevent evaporation. The plate was shaken using 600 rpm double orbital shaking mode at 45°C. ThT fluorescence was measured with 400 s interval with an excitation filter at 450 nm and an emission filter at 480 nm.

The ThT signal from the experiments was evaluated with respect to lag time and exponential phase rate constant. The lag time was defined as the time point where 10% of the maximum fluorescence is reached. The rate constant of the exponential phase was calculated using a fitting of the sigmoidal fibrillation curve in GraphPad Prism 4.0 based on the Finke-Watzky equation (34) and assuming that all insulin was converted to fibrils in the reaction.

**Stability of the Excipient**

The loss of soluble \(\alpha\)-crystallin and HSA was followed during the fibrillation of insulin using the settings from the well plate assay. To quantify the excipient during insulin fibrillation, the fluorescence signal of three wells was recorded, and the samples from these wells were taken out and analysed using HP-SEC as described below. This procedure was repeated at each time point.

**High Performance Size-Exclusion Chromatography**

The quantification of native protein fraction was performed on two different HP-SEC systems. The first one consisted of a 515 pump, a 717 autosampler (Waters, Milford, US) and a SPD UV/Vis detector (Shimadzu, Kyoto, Japan), using a TSK 3,000 SWXL column (Tosoh, Tokyo, Japan). The mobile phase used was 100 mM ammonium acetate, pH 6.8, with 0.3 M sodium chloride. The second system used consisted of an L7100 pump, an L7200 autosampler and an L7400 UV/Vis-detector (Merck-Hitachi, Darmstadt, Germany). The column used was a TSK 4,000 SWXL column (Tosoh, Tokyo, Japan) and the mobile phase a 50 mM phosphate buffer, pH 7.4, with 100 mM NaCl. No apparent difference in the protein quantification was observed with the two systems. For both systems 50–150 \(\mu\)l samples were injected, and separation was performed at a flow rate of 0.5 ml/min. The absorbance at 276 nm was recorded, and the areas of the peaks were determined.

**Fluorescence Spectroscopy**

Steady state fluorescence emission scans were collected on a FLS 920 spectrometer (Edinburgh Instruments Ltd., Livingston, UK) fitted with a plate reader. The samples from the formulations were diluted 5-fold. ThT was used in an end concentration of 5 \(\mu\)M and 4,4′-Bis(1-anilino-1-naphtalene-8-sulfonate) (bis-ANS) in an end concentration of 1 \(\mu\)M. ThT-containing samples were excited at 442 nm, and emission at 482 nm was recorded; bis-ANS excitation was at 385 nm, and the emission value at 488 nm was recorded. The values were accumulations of 3 measurements with a dwell time of 0.5 s and slit widths of 6 nm. The fluorescence signal was normalized to the value of an unstressed formulation containing bis-ANS.

**Circular Dichroism**

The experiments were performed on a JASCO J-815 circular dichroism spectrometer (Jasco, Tokyo, Japan). The insulin samples were scanned in a 1-cm quartz sample cell from 300 to 250 nm and in a 1-mm quartz sample cell between 240 and 195 nm, using a bandwidth of 2 nm, a response time of 2 s and a scan rate of 100 nm·min\(^{-1}\). Each spectrum was an average of three scans, and a reference scan of the relevant buffer was subtracted.

**UV Spectroscopy**

UV spectroscopy was performed on an 8,453 UV/VIS spectrometer (Agilent, Santa Clara, US). The spectra were obtained between 190 and 400 nm in a 10-mm quartz cell and corrected for the respective buffer signal.
Light Obscuration

The number and size of subvisible particles was measured using light obscuration, a method described in the European pharmacopeia (35). Light obscuration is based on the amount of light blocked as suspended particles pass a light beam. The measurements were performed on a SVSS-C system (PAMAS GmbH, Rutesheim, Germany) set with a pre-run volume of 300 µl and three measurements of 200 µl. The number of particles ranging in size from 1–50 µm was collected.

RESULTS

The insulin formulations were tested in two different fibrillation assays, both applying stirring to stress the formulations. One was performed in fluorescence well plates placed in a fluorescence plate reader for online measurement of fibrillation kinetics. The other one was performed in formulation vials and used for further characterization of the formulations with a range of analytical methods. In both assays, insulin is in contact with the liquid-air interface and glass and plastic surfaces, conditions, which are known to induce fibrillation (11,36).

Vial Assay

The vial assay was carried out in formulation vials with magnet stirring at three different temperatures (45°C, 25°C and 5°C), as described in experimental procedures. The three formulations were each incubated in triplicate, and samples taken at regular intervals were analyzed for ThT fluorescence and for native insulin recovery using HP-SEC. The HP-SEC method showed baseline-separated peaks of the three proteins in the formulations (Fig. 1) and can thus be used for quantification of non-fibrillated insulin and excipient.

Characterization at 45°C

Over the 8-hour time course of the stirring experiment at 45°C and in the absence of excipients, soluble insulin completely disappeared from the solution, as observed by HP-SEC. A corresponding rise in ThT signal suggests that the primary degradation product is fibrils (Fig. 2A). The presence of α-cristallin (0.2 mg ml⁻¹) completely prevented the formation of ThT-binding species. A small decrease (<10%) in the native insulin fraction was observed for all three vials, possibly due to formation of non-ThT-binding aggregates. Also, the use of HSA significantly limited the formation of ThT-binding species compared to the plain insulin formulation (Fig. 2A). However, the loss of native fraction was in the range of 10–30% over the 8-hour course of the experiment. ThT is generally considered selective for amyloid structure, but random aggregates with little or no ThT-binding capability can also be formed in the assay. Most aggregates, including fibrils, show affinity for bis-ANS, a commonly used probe for hydrophobicity (37). The bis-ANS fluorescence results (Fig. 3) showed that the insulin formulation contained physical degradation products interacting with bis-ANS. In contrast, the α-cristallin-containing insulin sample did not show bis-ANS-binding physical degradation products. This suggests that α-cristallin prevented the formation of random aggregates of insulin. Native HSA binds bis-ANS due to its hydrophobicity and gave a large background signal in our experiments. Thus, no measurements were carried out on the HSA-containing formulation.

Optical density of the formulations was measured at 350 nm (Fig. 4A), and subvisible particles were quantified using light obscuration (Fig. 4B). The unstressed formulations generally had an optical density of less than 0.04, and subvisible particle (<50 µm) counts were in the range 4–8×10³ particles ml⁻¹.

The stressed excipient-free insulin formulations showed an optical density of 2.2 as well as an increase in particle count to 2.5×10⁵ particles ml⁻¹. The formulations containing α-cristallin showed no significant increase in optical density, but an increase in particle count to 2.7×10⁴ particles ml⁻¹. The formulation containing HSA showed a substantial increase in both optical density (1.2) and particle count (2.2×10⁸ particles ml⁻¹).

In order to study whether the protein excipients themselves aggregate in the assay, two samples containing only α-cristallin or HSA were tested; both showed an increase in subvisible particle count (2.0×10⁷ and 1.4×10⁶ particles ml⁻¹, respectively). In addition, the HSA sample showed a moderate increase in optical density to 0.37.
Fig. 2 Physical degradation of insulin (0.58 mg ml$^{-1}$), in the absence of stabilizer or in the presence of α-crystallin (0.2 mg ml$^{-1}$) or HSA 2.5 mg ml$^{-1}$ in the vial assay as measured in triplicate (each line and symbol representing a replicate) by ThT fluorescence (upper panels) and HP-SEC (lower panels). The experiment was done at three temperatures: 45°C (A), 25°C (B) and 5°C (C). The observed standard deviations on three measurements of the same sample via both methods were less than 5%. The time scale is different between the panels.
Thus, the excipients also form aggregated products in the assay, with α-crystallin showing less particle formation than HSA.

Circular dichroism, in both the near-UV (NUV-CD) and the far-UV range (FUV-CD), was performed in order to determine the tertiary and secondary structure, respectively, of insulin and α-crystallin. The FUV-CD spectrum of the unstressed insulin formulation, with minima at 222 and 208 nm (Fig. 5A), is typical for α-helical structure. The NUV-CD spectrum shows a minimum at 276 nm (Fig. 5B). The intensity and shape of the latter signal is related to dimer and hexamer formation of insulin (38). The signal of α-crystallin was so small in both FUV-CD (Fig. 5C) and NUV-CD (Fig. 5D) that it could be subtracted from the signal of the whole formulation, yielding the signal of insulin alone. Thus, the insulin structure could not be determined by CD in the presence of HSA.

The stressed insulin formulations showed a substantial loss of α-helical structure, as indicated by the lack of the minima in the FUV-CD spectrum. The NUV-CD spectrum of the stressed formulation has no distinct features, and is typical of light scattering effects. In the presence of α-crystallin, no changes in the spectra were observed by FUV-CD (Fig. 5A), while the NUV-CD (Fig. 5B) showed a slight decrease in the (negative) signal. This suggests a slight loosening of the tertiary structure or dissociation of insulin, an effect which could be caused by α-crystallin, as we reported earlier (33). To further examine the stability of α-crystallin, spectra were taken of unstressed and stressed α-crystallin with no insulin present. Both in FUV-CD (Fig. 5C) and NUV-CD (Fig. 5D), only small changes in the spectra were observed, suggesting only minor perturbation of the α-crystallin structure.

**Stability Study at 25 and 5°C**

At 25°C, the experiment was performed for 32 h to obtain a reasonable amount of physical degradation. In the course of the experiment, the excipient-free insulin formulation lost 10–50% of the native fraction, with a corresponding increase in ThT fluorescence (Fig. 2B). The use of α-crystallin limited the loss of native insulin to between 0 and 10%, while HSA limited the loss to 5–20%, with corresponding increases in ThT signal observed for both formulations.

At 5°C, the experiment was carried out for 20 days (Fig. 2C). The loss of native fraction in the excipient-free insulin formulation was 35–50% with a corresponding rise in ThT fluorescence. No significant loss of soluble insulin in the formulation containing α-crystallin and no increase in ThT signal was observed. For the HSA-containing formulation, a loss of 15–50% of the native insulin fraction was observed, with corresponding increase in ThT signals.

These results show that α-crystallin also has a protective effect at lower temperatures, limiting the aggregation...
propensity of the insulin formulation to a higher degree than HSA.

Well Plate Assay

The insulin formulations with addition of varying concentrations of α-crystallin (0.02–0.2 mg ml$^{-1}$), HSA (1–5 mg ml$^{-1}$), sucrose (1–10% w/v) and polysorbate 80 (0.0075–0.03% w/v) were stressed by shaking at 45°C in a fluorescence well plate reader, and the formation of ThT-binding species was continuously monitored.

For all insulin formulations, the stress resulted in the appearance of ThT-binding species starting with a lag phase (constant low signal), followed by an exponential phase (signal increases exponentially), and ending in an equilibrium phase (constant high signal). Examples are shown for insulin with increasing amounts of α-crystallin and HSA in Fig. 6A & B, respectively. The binding of ThT, a probe selective for amyloid structures, and the characteristic sigmoidal shape of the fluorescence-time curve indicate that fibrils were formed (39). Moreover, insulin is known to fibrillate under comparable shaking stress conditions (10).
Control samples without insulin but with the excipients showed only a marginal ThT fluorescence signal and no increase during the course of the experiment (data not shown). Thus, no fibril formation was observed for the protein excipients.

The physical stability of the formulations was ranked using the parameter’s lag time and reaction rate in the exponential phase as described in experimental procedures. The lag time measured in the insulin formulation was 2.3±0.3 h, while the apparent reaction rate was 2.2±0.3 h^{-1} (Fig. 6 & Table I). Increasing α-crystallin concentrations progressively prolonged the lag time to 60±16 h at 0.2 mg ml^{-1} and also lowered the reaction rate to 0.4±0.1 h^{-1} (Fig. 6A,C & Table I). Higher concentrations of α-crystallin were tested, but no further increase in the lag time was observed (data not shown). For HSA, a low but statistically significant protective effect was obtained at concentrations of 2.5 mg ml^{-1} (lag time of 3.5±0.8 h) and higher (Fig. 6B,D & Table I). The reaction rate decreased significantly at all concentrations, with the lowest (0.5±0.1 h^{-1}) at 5 mg ml^{-1} (Table I). The use of 10% sucrose also yielded a small but significant increase in the lag time.

### Table 1

| Excipient  | Concentration (mg·ml^{-1}) | Approximate molar ratio (insulin: excipient)^a | Lag time (hours) | Exponential phase reaction rate (hours^{-1}) |
|------------|-----------------------------|-----------------------------------------------|------------------|-----------------------------------------------|
| None       | —                           | —                                             | 2.3±0.3          | 2.2±0.3                                       |
| α-crystallin | 0.02                        | 100 : 1                                       | 13±7.5*          | 0.8±0.3*                                      |
|            | 0.04                        | 50 : 1                                        | 28±16*           | 0.8±0.3*                                      |
|            | 0.08                        | 25 : 1                                        | 51±16*           | 0.5±0.2*                                      |
|            | 0.2                         | 10 : 1                                        | 60±16*           | 0.4±0.1*                                      |
| HSA        | 1.0                         | 7 : 1                                         | 2.7±0.2          | 1.1±0.2*                                      |
|            | 2.5                         | 3 : 1                                         | 3.5±0.8*         | 0.6±0.1*                                      |
|            | 5.0                         | 1 : 1                                         | 3.4±0.3*         | 0.5±0.1*                                      |
|            | 100                         | 1 : 300                                       | 2.4±0.2          | 1.9±0.6                                       |
| Sucrose    | 10                          | 1 : 300                                       | 2.5±0.1          | 2.1±0.3                                       |
|            | 50                          | 1 : 1,500                                     | 2.7±0.2*         | 1.9±0.1                                       |
|            | 100                         | 1 : 3,000                                     | 2.5±0.2          | 4.3±0.5*                                      |
|            | 0.075                       | 2 : 1                                         | 3.0±0.4*         | 3.2±0.5*                                      |
|            | 0.15                        | 1 : 1                                         | 3.0±0.4*         | 3.2±0.5*                                      |
| Polysorbate | 0.30                        | 1 : 2                                         | 3.4±0.3*         | 2.5±0.3*                                      |

^a The molar ratio is calculated based on monomeric form of the proteins.

*Statistically different from that of the insulin formulation without excipient with a 95% confidence limit using a student t-test.

**Fig. 7** The upper (A and B) show insulin (0.58 mg ml^{-1}) fibrillation as observed by fraction of soluble insulin by HP-SEC (●, solid line) and fraction of the maximum ThT signal (▼, dash dotted line). The fibrillation was performed in the presence of 0.01 mg ml^{-1} α-crystallin (A) and 2.5 mg ml^{-1} HSA (B). The soluble fraction of the excipients in the same experiment is shown in (C) and (D) for α-crystallin and HSA, respectively. The panels show the remaining excipient fractions in the presence of insulin (●, dotted line) and a control stress experiment where no insulin was present (▲, dashed line), as determined by HP-SEC. The error bars show the standard deviation of three separate samples. The lines are for guidance purposes only.
to $2.7\pm0.2\text{ h}$, while the use of polysorbate 80 yielded a significant increase in lag time both at $0.015\%$ and $0.03\%$, with the longest lag time ($3.4\pm0.3\text{ h}$) at $0.03\%$ (Table I). Neither polysorbate 80 nor sucrose significantly decreased the reaction rate, and polysorbate 80 surprisingly increased it, especially at the lowest concentration tested (Table I).

**Stability of the Excipient**

The concentration of the soluble fraction of protein excipients during insulin fibrillation was followed by HP-SEC during the course of a well plate experiment. The excipient concentrations were $0.01$ and $2.5\text{ mg ml}^{-1}$ for $\alpha$-crystallin and HSA, respectively. Insulin fibrillation in both experiments was observed after $3$–$5\text{ h}$ (Fig. 7A & B). The soluble fraction of $\alpha$-crystallin declined more rapidly in the presence of stressed insulin than in a control sample with no insulin present (Fig. 7C). As the $\alpha$-crystallin peak declined, new peaks were observed at smaller elution volumes, suggesting that initially soluble aggregates were formed (Fig. 8). The rapid decline in soluble insulin concentration as well as the rise in ThT signal only occurred when soluble $\alpha$-crystallin (or soluble aggregates) was no longer detected in the HP-SEC chromatograms (compare Fig. 7A & C). For HSA, a steady decline in peak area was observed with no apparent effect on the presence of stressed insulin (compare Fig. 7B & D). This suggests that $\alpha$-crystallin binds to and co-aggregates with a fibrillation-prone intermediate of insulin, whereas HSA probably does not (or only to a very limited degree). Furthermore, the presence of soluble $\alpha$-crystallin prevents the formation of ThT-binding species, whereas HSA does not (Fig. 7).

**DISCUSSION**

We have investigated the effect of the molecular chaperone $\alpha$-crystallin as an anti-fibrillation excipient, compared to HSA, polysorbate 80 and sucrose.

The use of $\alpha$-crystallin in the insulin formulation increased the lag time of insulin fibrillation up to 26-fold for the highest concentration used (0.2 mg ml$^{-1}$). In contrast, polysorbate 80, sucrose and HSA, even at higher concentrations, only increased the lag time by a factor of 1.5, 1.2 and 1.5, respectively (Table I). Even the lowest concentration of $\alpha$-crystallin used (0.02 mg ml$^{-1}$) provided a 6-fold prolongation of the lag time. The stirring stress method in vials at $45^\circ\text{C}$ showed some formation of particles in the $\alpha$-crystallin-containing formulation in the subvisible range, but no increase in optical density (Fig. 4). Our structural data (Fig. 5) on the stressed vial formulations show little or no perturbation of the insulin structure when $\alpha$-crystallin was present. In contrast, significant loss of structure of insulin was observed in the excipient-free formulation. Also, control samples containing only $\alpha$-crystallin showed little loss of structure after $8\text{ h}$ incubation.

HSA showed a clearly lower protective effect (Fig. 2) and a high increase in particle count and optical density (Fig. 4). The latter suggest that HSA itself aggregated in the assay. The results from our vial assay at 5 and $25^\circ\text{C}$ also showed that the stabilizing effect of $\alpha$-crystallin is not limited to high temperatures, even though its chaperone-like capabilities have been reported to increase above physiological temperature (19,24,25). At all temperatures, the $\alpha$-crystallin-containing formulation outranked the other formulations, both in terms of fibrils formed and remaining native fraction at the end of the experiment (Fig. 2). In conclusion, the effect of $\alpha$-crystallin in both assays was far superior to that of all other excipients used.

The common excipients used in the assays stabilize insulin through various mechanisms. The non-ionic surfactant polysorbate 80 and HSA probably prevent insulin adsorption to container surfaces and the air–water interface through preferential adsorption (1,29). Protein adsorption to interfaces is known to promote formation of partially folded protein intermediates. Thus, a reduced adsorption of insulin lowers the formation rate of fibrillation-prone species. The excipient sucrose is preferentially excluded from the protein surface domain and thereby reduces the population of partly denatured and misfolded species (40). In addition, the preferential exclusion effect will stabilize higher association states of insulin, i.e., the hexamer and dimer, but also fibrils. The common excipients are thus all expected to reduce the accumulation of partially folded intermediates and nuclei, albeit through different mechanisms. Nonetheless, only a very marginal protective effect of the excipients, compared to $\alpha$-crystallin, was
observed using excipient concentrations normally found in protein formulations.

The exponential phase rate constants in formulations containing α-crystallin or HSA decreased about 4-fold (Table I). Thus, both excipients have a moderate effect on the elongation of fibrils. As both proteins are reported to interact directly with proteins (23,29), this effect could be due to association with intermediate species, thus delaying their addition to fibrillar species. However, a direct interaction with the growth site of the fibrils cannot be excluded based on our data.

Soluble α-crystallin, but not HSA, disappears from the solution prior to the fibrillation of insulin (Figs. 7 & 8). This indicates the formation of eventually insoluble co-aggregates with pre-fibrillar insulin species. This is in agreement with recent studies on the mechanism of suppression of amyloid fibrillation by small heat shock proteins (26,27,41). Dual polarization interferometry studies have shown that inhibition of α-synuclein fibrillation by α-crystallin occurs prior to formation of ThT-binding species (26). A mechanism of amyloid fibrillation inhibition has recently been suggested in a review by Ecroyd & Carver (42), based on the nucleation-dependent polymerization model of fibrillation (43). It describes the binding by small heat shock proteins to occur to all non-native fibrillation-prone species, including mono/dimeric intermediates and fibrillation nuclei. This is plausible, as α-crystallin is suggested to be specific to partially denatured or misfolded proteins (24), descriptions that fit well with the proposed structure of the intermediate species in the insulin fibrillation pathway (10,12). Fibrillation nucleation is described as a stochastic event, where one or only a few nuclei are formed (13). Subsequently, autocatalysis (secondary nucleation) through the first formed fibrils (13) results in the conversion of bulk native insulin to insoluble fibrils. Inhibition at this critical step in fibrillation could account for the increase in insulin stability observed in our assays. In conclusion, the direct interaction with intermediates leading to the formation of the critical fibrillation nucleus is most likely responsible for the superior effect of α-crystallin compared to all other excipients.

We have demonstrated that α-crystallin is an effective inhibitor of fibrillation in a formulation of the therapeutic protein insulin. Expression and purification of recombinant human α-crystallin for excipient use is feasible and can be expected to have similar activity to the bovine α-crystallin tested in this work (44,45). There are, however, several unknown factors that may oppose the commercial use of α-crystallin as an excipient. First, several members of the small heat shock family have been implicated in immuno-activating effects (46–48), thus potentially stimulating unwanted immunological reactions towards the therapeutic protein. Second, its long-term stability is as yet unknown. However, since it is effective in very low concentrations the presence of α-crystallin degradation products may not be a significant problem, as long as functionality is retained. Third, the interaction with other potential excipients, e.g., phenolic preservatives, is unknown. Binding of these compounds to α-crystallin, and subsequent loss of activity of either, will need to be determined. Finally, α-crystallin may be problematic in some analytical assays, due to interference. However, (recombinant) HSA is currently in use as a stabilizing additive in some protein formulations at much higher concentrations than those that would be required for α-crystallin.

CONCLUSIONS

The molecular chaperone α-crystallin shows a superior stabilizing effect on insulin compared to the other tested excipients, HSA, polysorbate 80 and sucrose. The favorable stabilizing action of α-crystallin is proposed to be due to specific interaction between α-crystallin and all non-native insulin species involved in the fibrillation process. This interaction delays formation of the fibrillation nucleus and thus the physical degradation of the bulk protein. This makes small heat shock proteins interesting candidates as excipients in formulations of insulin and other therapeutic peptides and proteins with propensity to fibrillate.

ACKNOWLEDGEMENTS

The authors thank Dr. John den Engelsman, Dr. Minna Groenning and Stefan Romeijn for their kind help and suggestions, and Schering-Plough for the kind gift of insulin. We also acknowledge the Alfred Benzon Foundation and the Drug Research Academy for funding the Fluostar plate reader. TR is grateful for travel grants from the Oticon Foundation and Knud Højgaard’s Foundation and for a Drug Research Academy International PhD grant.

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