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Effects of confinement on insulin amyloid fibrils formation

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Abstract Insulin, a 51-residue protein universally used in diabetes treatment, is known to produce amyloid fibrils at high temperature and acidic conditions. As for other amyloidogenic proteins, the mechanisms leading to nucleation and growth of insulin fibrils are still poorly understood. We here report a study of the fibrillation process for insulin confined in a suitable polymeric hydrogel, with the aim of ascertain the effects of a reduced protein mobility on the various phases of the process. The results indicate that, with respect to standard aqueous solutions, the fibrillation process is considerably slowed down at moderately high concentrations and entirely suppressed at low concentration. Moreover, the analysis of the initial stages of the fibrillation process in aqueous solutions revealed a large spatial heterogeneity, which is completely absent when the fibrillation is carried out in the hydrogel. We attribute this heterogeneity to the diffusion in solution of large amyloid aggregates, which must be formed very fast compared to the average times for the whole sample. These findings are interpreted in the framework of recently suggested hetero-

geneous nucleation mechanisms. Moreover, they may be useful for the development of new insulin pharmaceutical formulations, more stable against adverse conditions.

Keywords Insulin · Amyloids · Poly vinyl-alcohol · Thioflavin T

Introduction

The understanding of the mechanisms leading to the formation of protein ordered aggregates (amyloid fibrils) is at the moment one of the major challenges in biophysical and biomedical research. In fact, the formation of amyloid fibrils is involved in a number of pathologies such as Alzheimer's and Parkinson's disease (Harper and Lansbury 1997; Dobson 2003; Uversky and Fink 2004). The propensity to form amyloid aggregates, rather than being restricted to disease-related proteins, seems to be a generic property of polypeptide chains (Fandrich and Dobson 2002). Under appropriate destabilizing conditions, even globular, otherwise stable proteins have been shown to self-assemble into amyloid fibrils (Fandrich et al. 2001). Protein conformational changes have been recognized to play a key role in the pathways leading to amyloid formation (see e.g. Uversky and Fink 2004) and in general to protein aggregation (Militello et al. 2003, 2004; Vetri and Militello 2005).

At low pH and high temperature insulin is very prone to form amyloid fibrils (Waugh 1946; Burke and Rougvie 1972; Yu et al. 1974; Bouchard et al. 2000). In the absence of agitation, the kinetics of the fibrillation process display a sharp time dependence, with a

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pronounced lag phase and a subsequent explosive growth of aggregates (Nielsen et al. 2001a, b). In analogy with what observed for sickle-cell hemoglobin polymerization (Ferrone et al. 1985; Ferrone 1999), it has been recently shown that such time dependence can be rationalized by means of heterogeneous nucleation mechanisms, i.e. mechanisms by which a pre-existing fibril may promote the formation of new fibrils (Librizzi and Rischel 2005). The existence of such mechanisms is in agreement with time-lapse atomic force microscopy (AFM) measurements, showing that in the early stages of the aggregation process insulin fibrils display the tendency to grow close to each other (Jansen et al. 2005). Heterogeneous nucleation gives to the process its characteristic exponential growth behavior, since the rate of formation of new fibrils depends on the amount of fibrils already present in the sample. Analogous phenomena have been observed in the fibrillation process of islet amyloid polypeptide (Padrick and Miranker 2002; Koo and Miranker 2005) and glucagon (Pedersen et al. 2006a, b). On the contrary, such cooperative mechanisms are not observed for other proteins, when the fibrillation process is likely to proceed only by tip-to-tip association (Carrotta et al. 2005; Vetri et al. 2007). For insulin, the abrupt time dependence of amyloid aggregation has been also attributed to lateral growth of fibrils (Manno et al. 2006, 2007), as originally suggested by Waugh (Waugh et al. 1953; Waugh 1957).

The existence of heterogeneous nucleation mechanisms could in principle bring about some kind of spatial heterogeneity in the fibrillation process (Ferrone 1999). Moreover, it implies a peculiar role for the diffusional properties of the samples under investigation, since the diffusion of already fibrillated proteins may propagate the amyloid aggregation also in other regions of the sample. In this context, a study of insulin fibrillation in a suitable confined environment can help the understanding of the basic mechanisms of the process. On different scales, the effects of confinement on the behaviour of proteins are a subject of interest also in other fields of protein research, such as protein folding (see e.g. Hayer-Hartl and Milton 2006) and protein dynamics (see e.g. Schirò et al. 2005).

We studied the fibrillation process of insulin embedded in a polymeric hydrogel (Poly vinyl-alcohol, PVA), often used as a drug delivery system (Hassan and Pappas 2000; Cascone et al. 2002), and compared the results with those obtained in aqueous solutions in analogous experimental conditions. The formation of amyloid fibrils was monitored in situ by means of Thioflavin T (ThT) fluorescence (see “[Materials and methods](#)”), both in solution and in PVA hydrogels; in this

latter case, the dye was embedded into the hydrogel together with the protein. Insulin aggregation is known to be sizably accelerated by the presence of hydrophobic surfaces (Sluzky et al. 1991, Sharp et al. 2002). Due to the hydrophilic nature of PVA hydrogels (Cascone et al. 2002), in our system no effects are expected on the fibrillation process other than those brought about by the reduced mobility of protein molecules and aggregates.

Materials and methods

All experiments were performed at $T = 60^\circ\text{C}$, pH 1.6, HCl 0.025 M, NaCl 0.1 M. Bovine insulin and ThT were purchased from Sigma and used without further purification. All samples were freshly prepared, centrifuged and filtered through 0.22 μm filters. ThT was prepared in a stock solution (1 mg/ml) and stored at 4°C protected from light to avoid quenching. Before each experiment, aliquots of this stock solution were added to the insulin solutions, to a final ThT concentration of 10 $\mu\text{g/ml}$ (in situ procedure). ThT is widely used as a selective probe for the detection of amyloid fibrils, since in the presence of fibrils it brightly fluoresces, with excitation and emission maxima at approximately 450 and 482 nm, respectively (Naiki et al. 1989; Levine 1999; Krebs et al. 2005).

PVA (Fluka, MW 47000) aqueous solutions were obtained at 80°C by dissolving 4 g of PVA in 100 ml of deionized water. To produce PVA hydrogels, the resulting solutions were γ -irradiated under N_2 atmosphere using a ^{60}Co γ -source; γ -irradiation was performed at 5°C with a 2 kGy/h dose rate and a total absorbed dose of 40 kGy. Work is in progress to characterize the internal topology of the obtained PVA hydrogels as a function of the γ -irradiation dose. A procedure was developed to cut standard portions of gel with a thickness of 3 mm and a surface of 0.2 cm^2 . These portions were loaded by equilibrium swelling in the solution containing insulin and ThT, at 4°C for ~ 15 . In order to protect the gel from dehydration at 60°C , experiments were performed by keeping the uploaded gel in the same solution used to dissolve insulin. Protein diffusion outside the gel was prevented by means of a dialysis membrane.

Fluorescence spectra were carried on a Jasco FP-6500, equipped with a Jasco ETC-273T peltier. No stirring was used and in all the cases the fibrillation process was allowed to proceed directly into the instrument, to avoid any kind of sample agitation. Spectra were collected at regular intervals (3 or 4 min), with excitation at 450 nm, emission and excitation bandwidth 3 nm,

scan speed 100 nm/min and integration time 1 s. Solution experiments were performed in standard fluorescence cell (1 cm × 1 cm). For PVA samples, a suitable sample holder was designed to keep the small portions of loaded hydrogel wrapped in the dialysis membrane at the centre of the fluorescence beams, into a standard 1 cm fluorescence cell. Afterwards, fluorescence measurements were performed in back-scattering geometry.

Protein concentration was determined spectrophotometrically both in solution and after loading into the gel.

Results and discussion

Figure 1 shows the ThT fluorescence as a function of time at two different protein concentrations, both for aqueous solutions and PVA hydrogels. In agreement with previously reported data (Nielsen et al. 2001a), in aqueous solution the kinetics exhibit a long lag phase and a subsequent very fast growth of amyloid aggregates, as revealed by ThT fluorescence. Since the uptake of the protein in PVA hydrogels required ~15 h at 4°C (see “Materials and methods”), we also report for comparison the kinetics obtained in aqueous solution in the same experimental conditions, but after 15 h at 4°C. In PVA hydrogels, the fibrillation process is sizably slowed down at higher concentration (Fig. 1b) and entirely suppressed at low concentration (Fig. 1a). These observations in principle can help the development of insulin pharmaceutical formulations, more stable against adverse conditions.

It must be noted that for other proteins the fibrillation process in the presence of crowding agents is often reported to be significantly accelerated, due to excluded volume reasons (see, e.g. Munishkina et al. 2004).

In our case, the slowing down at the higher concentration (Fig. 1b) can be explained by a corresponding slowing down of all the diffusive processes within the hydrogel, which influences the kinetics of the fibrillation. The suppression of fibrils formation at low concentration (Fig. 1a) cannot be explained by the slowing down of diffusive processes. Indeed, this should simply slow down the process, as for the sample at higher concentration. In analogy with what has been suggested for sickle cell hemoglobin polymerization, insulin fibrillation could be promoted by large concentration fluctuations, related to the thermodynamic instability of the protein solution (Vaiana et al. 2003). In PVA hydrogels, concentration fluctuations would be strongly inhibited, and therefore at low concentration the process could be even completely suppressed. A peculiar role for local concentration fluctuations in the initial stages of insulin fibrillation has been recently suggested (Podestà et al. 2006). It must be noted, however, that no thermodynamic instability has never been reported for insulin solutions, a part from, of course, the aggregation by itself.

Figure 2 reports the ThT fluorescence spectra during the progress of the fibrillation process in aqueous solutions, for the samples at 0.8 mg/ml (a and b) and 2.0 mg/ml (c and d), both for freshly prepared solutions (left panels) and after 15 h at 4°C (right panels). As can be seen, some of the spectra result remarkably distorted, especially for fresh solutions. These spectra were recorded immediately at the end of the lag phase, and are paralleled by fluctuations in the Rayleigh scattering of the fluorescence excitation beam (data not shown). These observations, although qualitative, indicate the presence in solution, just at the end of the lag phase, of amyloid aggregates producing fluctuations on the fluorescence signal when crossing the excitation beam. For the freshly prepared

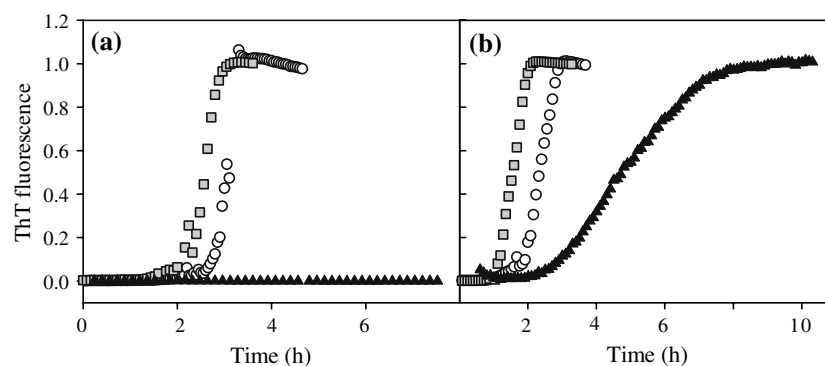
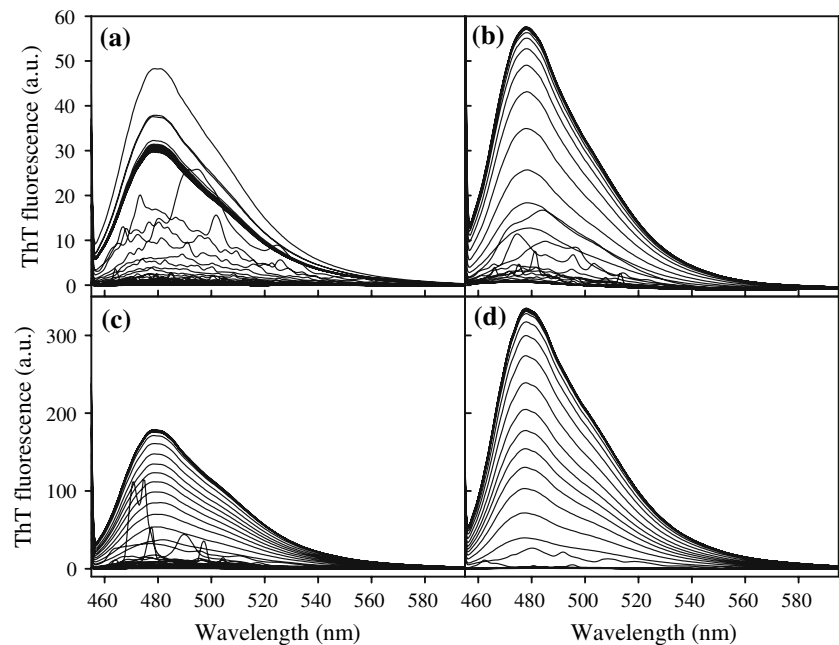


Fig. 1 ThT fluorescence as a function of time during the fibrillation process in aqueous solutions and in PVA hydrogels, at two different protein concentration. **a** 0.8 mg/ml; **b** 2.0 mg/ml. Data were normalized to the final value of the signal. *Circles* fresh solu-

tion, *Squares* solution after 15 h at 4°C, *Triangles* in PVA hydrogels. For the sample at 0.8 mg/ml in PVA hydrogel (*panel a*), no fibrillation was detected up to 12 h

Fig. 2 ThT fluorescence spectra during the fibrillation process. **a** 0.8 mg/ml fresh solution, **b** 0.8 mg/ml after 15 h at 4°C, **c** 2.0 mg/ml fresh solution, **d** 2.0 mg/ml after 15 h at 4°C. No signal fluctuations were detected for the sample at 2.0 mg/ml in PVA hydrogel



samples (Fig. 2a, c), such early aggregates must be considerably large, since the amplitude of the fluctuations in the fluorescence signal is comparable with the final average value. It is important to note that no fluctuations at all were observed when the fibrillation process was brought about in PVA hydrogels (not shown). Data above reported indicate that insulin fibrillation is characterized by a considerable spatial heterogeneity, especially in the early stages of the process, in full agreement with the existence of secondary nucleation pathways (Librizzi and Rischel 2005). In a given sample region, the formation of the first stable amyloid aggregates can be considered an inherently stochastic process (Ferrone 1999). These aggregates suddenly promote the formation of other fibrils, thus leading in the interested region to a concentration of fibrillated proteins much larger than the average concentration in the whole sample. In solution, these early large aggregates may diffuse in the sample. When they cross the fluorescence excitation beam, a strong fluctuation is observed in the measured ThT fluorescence. Large-scale diffusive processes are clearly suppressed when the fibrillation is brought about in PVA hydrogels, and therefore in this case no fluctuation is observed in the ThT fluorescence. Signal fluctuations were also observed for the samples in aqueous solution after 15 h at 4°C (Fig. 2b, d). In the presence of secondary nucleation pathways, the diffusion of amyloid aggregates in aqueous solution is likely to promote fibrils formation in other spatial regions, and this speeds up the fibrillation of the whole sample. In PVA hydrogels, this process is

restricted only to the regions accessible to the aggregates, and therefore the overall fibrillation process is slowed down.

In conclusion, data here reported support the idea that heterogeneous nucleation pathways are in fact extremely important in the global behavior of insulin fibrillation. Embedding the protein in a polymeric biocompatible hydrogel strongly inhibits large scale diffusive processes, thus making heterogeneous nucleation pathways less effective. This results in a considerable slowing down of the fibrillation process.

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