The non-ionic detergent Brij 58P mimics chaperone effects

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Abstract The non-ionic detergent Brij 58P is recommended as a stabilizing agent for protein storage; for example, the aggregation-prone chaperone DnaJ can be maintained in solution by low concentrations of Brij 58P. During protein folding studies with α-glucosidase, rhodanese and citrate synthase as model proteins, we discovered that the low concentrations of Brij 58P usually added with purified DnaJ to renaturation samples are sufficient to mimic chaperone effects with respect to prevention of protein aggregation. Furthermore, addition of Brij 58P to refolding α-glucosidase and citrate synthase enhanced the yield of refolded protein by a factor of two. Thus, Brij 58P can mimic chaperone effects and care should be taken when the substance is used to stabilize chaperone preparations.

Key words: Brij 58P; Chaperone; Protein aggregation; Protein folding

1. Introduction

Protein aggregation is considered the main culprit for low yields of recombinant protein in biotechnological processes. Unless efficient renaturation protocols can be established, aggregation and inclusion body formation can be a major obstacle to obtaining a given protein in the functional form. In general, protein aggregation is the consequence of mainly intermolecular hydrophobic interactions that arise prior to proteins attaining their native structures, where hydrophobic segments are normally buried in their interior. Thus, protein aggregation and inclusion body formation are often observed upon high recombinant expression of heterologous proteins. Inclusion body formation is the rule when heterologous proteins cannot attain their native structures, e.g. cytosolic expression of proteins that are disulfide-bonded in their native conformation.

Considering the natural intracellular molecular crowding in a cell with protein concentrations as high as 300–400 mg/ml, natural structure formation would appear to be a miracle [1]. It is generally assumed that in vivo prevention of aggregation and stimulation of structure formation is largely due to the action of molecular chaperones that transiently interact with non-native polypeptide segments and thereby keep them in folding-competent conformations [2].

In vitro refolding of inclusion body material is mostly carried out in the absence of chaperones. Considerable progress has been made by the discovery that certain low molecular weight compounds assist in vitro structure formation. These substances reduce aggregation of partially structured folding intermediates prone to collapse. The low molecular weight compound L-arginine, for example, has been recognized as suppressing aggregation during refolding and thus stimulating structure formation [3,4]. Another way to avoid precipitation is to utilize detergents that enhance solubility of hydrophobic folding intermediates.

Here we show via light-scattering experiments that the non-ionic detergent Brij 58P inhibits aggregation of chemically denatured model proteins at concentrations as low as 0.0001%. Furthermore, Brij 58P enhances the yield of refolded α-glucosidase and citrate synthase. Thus, Brij 58P efficiently mimics chaperone effects and may be a useful storage additive by impairing protein aggregation.

2. Materials and methods

2.1. Light-scattering studies

Protein aggregation was assayed by light scattering in a Hitachi F4500 fluorimeter equipped with a magnetic stirrer. Excitation and emission wavelengths were 500 nm, slit widths were 2.5 and 5 nm, respectively. Voltage was set to 700 V and as response time 2 s was chosen. As model proteins, highly purified recombinant, lyophilized α-glucosidase from *Saccharomyces cerevisiae* (a gift from Roche Diagnostics), rhodanese (bovine liver, Sigma) and citrate synthase (pig heart, Roche Diagnostics) were employed. Concentrations of refolding proteins giving rise to significant light scattering were 140 nM (9.52 μg/ml) for α-glucosidase, 500 nM (16.6 μg/ml) for rhodanese, 150 nM (7.74 μg/ml) for citrate synthase. The model proteins α-glucosidase and rhodanese were directly denatured at a concentration of 14 μM and 50 μM, respectively, in 5–5.5 M guanidinium hydrochloride, 50 mM Tris–HCl, 20 mM dithiothreitol (DTT), pH 8.0. Citrate synthase was dialyzed against 50 mM Tris–HCl, 2 mM EDTA, pH 8.0 before denaturation in 4.5 M guanidinium chloride, 50 mM Tris–HCl, 20 mM DTT, pH 8.0 at a protein concentration of 15 μM. Denaturation was performed for 2–4 h. At the beginning of each experiment the basic light-scattering signal of filtered folding buffer (50 mM KCl, 10 mM MOPS, pH 7.6) was recorded for 2 min. Aggregation-induced increase of light-scattering signals was then tested by diluting denatured proteins 1:100 into folding buffer to the final concentrations indicated above. Light scattering was monitored for 8 min. Brij 58P (Fluka), CHAPS (Biomol) and Triton X-100 (AppliChem) were added to folding buffer 1 min before addition of the model proteins.

2.2. Reactivation of α-glucosidase and citrate synthase

Reactivation of α-glucosidase after chemical denaturation was determined as described in [5]. A 440 μM (30 mg/ml) stock solution of α-glucosidase was prepared from lyophilized material and dialyzed overnight against reactivation buffer (10 mM potassium phosphate, 1 mM EDTA, pH 7.5). After dialysis, the enzyme was diluted 1:27-fold into 7 M guanidinium chloride, 50 mM Tris–HCl, 2 mM DTT, pH 8.0 to a final concentration of 14 μM (0.95 mg/ml) and incubated for at least 2 h at 22°C. Denatured protein was then diluted 1:100 into reactivation buffer containing 0.002% Brij 58P when indicated. Re-

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covery of enzyme activity was tested by the conversion of *p*-nitrophenol-α-glucoside (Sigma) to *p*-nitrophenol and glucose, of which the first is spectroscopically detectable at 405 nm. At defined time intervals, aliquots of the refolding sample were removed and diluted 1:100 into prewarmed assay buffer (100 mM potassium phosphate, pH 6.8) that contained the substrate *p*-nitrophenol-α-glucoside at a concentration of 2 mM. The kinetics of formation of *p*-nitrophenol was spectroscopically recorded for 1 min. Measurements were performed at 30°C.

For testing the reactivation of citrate synthase, the ammonium sulfate-stabilized suspension (190 μM) was first centrifuged at 13 000 rpm in a bench centrifuge for 10 min. The precipitate was dissolved in and dialyzed against 50 mM Tris–HCl, 2 mM EDTA, pH 8.0. For denaturation, the protein was diluted 1:9 into 7 M guanidinium chloride, 50 mM Tris–HCl, pH 8.0, 2 mM DTT. The final concentration of the enzyme in the denaturation buffer was determined by UV-absorption as 30 μM. For reactivation, the enzyme was diluted 1:100 into reactivation buffer (50 mM Tris–HCl, 2 mM EDTA, pH 8.0) containing 0.0002% Brij 58P when indicated. Reactivation yields were determined by measuring the enzyme activity after a 1:50 dilution into 50 mM Tris–HCl, 2 mM EDTA, 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 10 mM oxaloacetate, 15 mM acetyl CoA, pH 8.0. Activity determinations were performed at 412 nm at 25°C for 1 min.

3. Results and discussion

3.1. Brij 58P prevents aggregation of several unfolded proteins

Since earlier studies demonstrated that DnaJ in the absence of its cognate Hsp70 suppresses protein aggregation, the protein has been classified as an ATP-independent chaperone [6,7]. During our structure function studies of DnaJ domains, a classical diagnostic assay for DnaJ chaperone activity was performed: chemically denatured proteins were added to a buffer that contained DnaJ, and the capability of the chaperone to suppress aggregation was investigated via light-scattering [6,7]. As recommended in the literature we added the non-ionic detergent Brij 58P at a concentration of 0.05% as a stabilizer to our DnaJ preparations [8]. As DnaJ was diluted 1:100 from (Brij 58P-containing) storage buffer into the assay buffer to determine protein aggregation, final Brij 58P concentrations in the range of 0.0005% were tested for their effect on protein solubility. As a first model protein α-glucosidase was tested. The enzyme was denatured as indicated in Section 2 and then diluted in refolding buffer. Rapid dilution into refolding buffer resulted in significant aggregation. Addition of Brij 58P to final concentrations as low as 0.00002% (w/v) resulted in a clearly visible suppression of aggregation (Fig. 1A). Increasing the Brij 58P concentration to 0.002% (w/v) reduced the amplitude of light-scattering signals by more than 80%.

In order to test whether the observed effect of Brij 58P on the solubility of α-glucosidase may also hold true for other test proteins, rhodanese and citrate synthase were used as additional model proteins. With rhodanese, a Brij 58P concentration of 0.0005% reduced light scattering to about 60% of the signal observed in the absence of the detergent (Fig. 1B). Brij 58P concentrations as low as 0.001% resulted in a decrease of the amplitude to 40%. However, no considerable further suppression was achieved upon addition of Brij 58P concentrations higher than 0.002%. Only at a final concentration of 0.2% Brij 58P total suppression of the light-scattering signals was observed in case of rhodanese (data not shown).

As a third model protein citrate synthase was tested. The enzyme was diluted to a final concentration of 150 nM from denaturant into refolding buffer. While in the absence of Brij 58P aggregation-caused light-scattering was observed, a Brij 58P concentration of 0.0001% reduced the signal to less than 10% of the amplitude observed in the absence of Brij 58P (Fig. 1C). In order to test whether other detergents may have similar strong effects on the solubility of citrate synthase, CHAPS and Triton X-100 were tested. Concentrations of 0.1% CHAPS and 0.001% Triton X-100 reduced light-scattering signals to 40 and 70%, respectively. Thus, among the

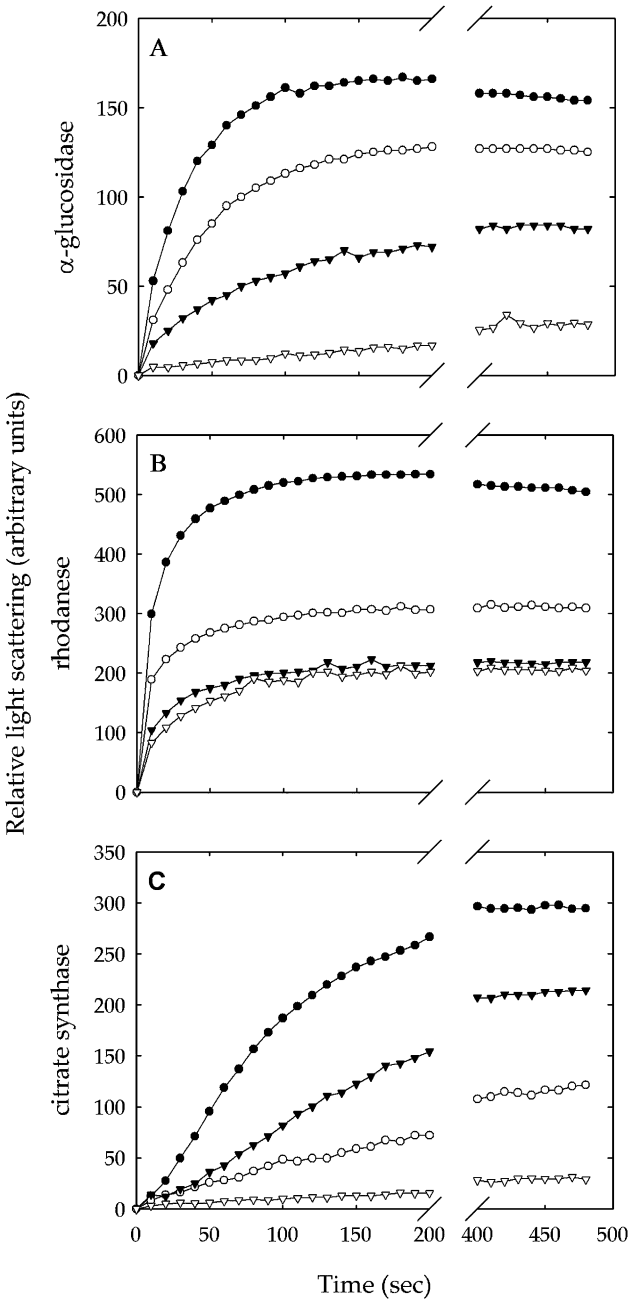

Fig. 1. Light-scattering experiments with chemically denatured model proteins. Light scattering was performed as indicated in Section 2. A: α-glucosidase in the absence of Brij 58P (filled circles), and in the presence of 0.00002% (open circles), 0.0002% (filled triangles) and 0.002% (open triangles) Brij 58P. B: Rhodanese in the absence of Brij 58P (filled circles) and in the presence of 0.0005% (open circles), 0.001% (filled triangles) and 0.002% (open triangles) Brij 58P. C: Citrate synthase in the absence of detergent (filled circles), in the presence of 0.1% CHAPS (open circles), 0.001% Triton X-100 (filled triangles) and 0.0001% Brij 58P (open triangles).

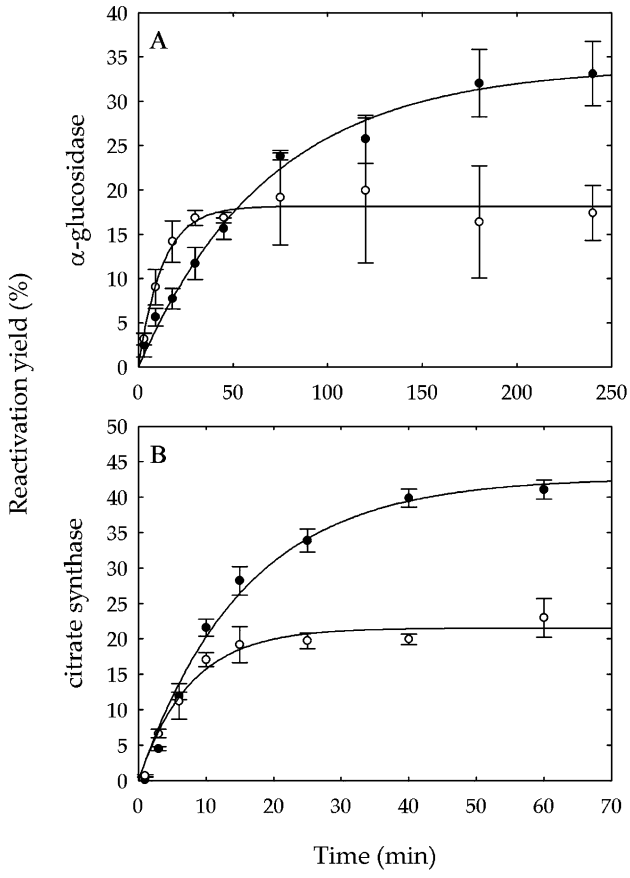

Fig. 2. Effect of Brij 58P on the reactivation of α-glucosidase and citrate synthase. A: Chemically denatured α-glucosidase was diluted to a final concentration of 140 nM into reactivation buffer without (open circles) and with (filled circles) 0.002% Brij 58P. Enzyme activity was determined as described in [5]. B: Citrate synthase was tested as indicated in Section 2. Citrate synthase was refolded at a concentration of 300 nM either in the absence (open circles) or the presence of 0.0002% Brij 58P (filled circles). Enzyme activity was tested as described in Section 2. Error bars indicate the standard deviations of three independent experiments. Data were fitted with the Sigma Plot 8.0 program.

detergents compared here, Brij 58P was the most powerful substance with regard to conferring solubility.

Calculating the molar ratios of denatured proteins and Brij 58P at the measured effective concentrations, surprisingly low molar ratios of Brij 58P support solubility of folding intermediates. Brij 58P concentrations that reduced the light-scattering signals to the minimum levels shown in Fig. 1 were 0.002% for α-glucosidase, 0.002% for rhodanese and 0.0001% for citrate synthase, corresponding to molar ratios of 130:1, 36:1 and 6:1 (Brij 58P:model protein), respectively. One could speculate that the Brij 58P concentration required to reduce aggregation may be determined by the relative hydrophobicities of the folding intermediates.

3.2. *Brij 58P enhances the refolding yields of α-glucosidase and citrate synthase*

In order to test whether Brij 58P may also enhance the yield of refolded protein, denatured α-glucosidase was diluted into reactivation buffer. The activity of α-glucosidase was tested after different time intervals. In the absence of Brij 58P, a rapid rise of enzyme activity was observed that reached a plateau after 50 min (Fig. 2A). Here, a maximum reactivation yield of ca. 18% and folding rate constants of 0.094 ± 0.04 min^{-1} were observed. Supplementation of the reactivation buffer with 0.002% Brij 58P (the concentration that suppressed aggregation of α-glucosidase to less than 10% in the light-scattering studies) resulted in a two-fold increase of the reactivation yield (44%) and a seven-fold decrease of the reactivation kinetics (0.014 ± 0.003 min^{-1}). With citrate synthase, a refolding yield of 22% was obtained in the absence of Brij 58P. Refolding rate constants were 0.17 ± 0.03 min^{-1} (Fig. 2B). The presence of 0.0002% Brij 58P in the refolding buffer caused a two-fold increase of the refolding yield and a two-fold decrease (0.078 ± 0.006 min^{-1}) of the rate constants.

These results indicate that the detergent Brij 58P mimics the action of molecular chaperones by suppressing aggregation and assisting structure formation. A similar beneficial effect on the refolding yield of α-glucosidase was observed earlier with GroE [5]. In that case, however, GroE did not significantly influence the refolding kinetics. Furthermore, the increase in yield caused by GroE was only ca. 30%. Notably, with Brij 58P renaturation yields for both enzymes were doubled. The slower folding kinetics caused by Brij 58P could be due to a slow dissociation of the denatured protein from the detergent. Alternatively, Brij 58P could act as a denaturant. The effect of denaturants on folding and unfolding rates is commonly depicted by Chevron plots in which folding kinetics are retarded at low concentrations of the denaturant, whereas above a critical threshold concentration the unfolding rates increase with the increasing concentration of denaturant giving a typical v-shaped curve (for a comprehensive review on protein folding see [9]). Certainly, a further analysis is required to analyze on a molecular level the effect of Brij 58P on protein folding and stability.

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