Solubilization of aggregated proteins by ClpB/DnaK relies on the continuous extraction of unfolded polypeptides

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Abstract The AAA+ chaperone ClpB solubilizes in cooperation with the DnaK chaperone system aggregated proteins. The mechanistic features of the protein disaggregation process are poorly understood. Here, we investigated the mechanism of ClpB/DnaK-dependent solubilization of heat-aggregated malate dehydrogenase (MDH) by following characteristics of MDH aggregates during the disaggregation reaction. We demonstrate that disaggregation is achieved by the continuous extraction of unfolded MDH molecules and not by fragmentation of large MDH aggregates. These findings support a ClpB-dependent threading mechanism as an integral part of the disaggregation reaction.
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1. Introduction

The survival of eubacteria, plants, yeast cells and mitochondria at extreme temperatures depends on the ability to reverse stress-induced protein aggregation [1–4]. This activity is mediated by the ring-forming AAA+ chaperone ClpB/Hsp104 that cooperates with an Hsp70 chaperone system (e.g., the bacterial DnaK system (KJE)) to solubilize and refold aggregated proteins [5–11].

Using malate dehydrogenase (MDH) as a thermolabile model substrate, the refolding of aggregated proteins could be separated into two phases [6]. Aggregated proteins are first solubilized by the combined action of ClpB and KJE, and subsequently refolded by the KJE system alone [6]. The precise mechanism of the initial disaggregation reaction is still unknown. It has been suggested that ClpB interacts with protein aggregates first, thereby mediating changes in the aggregate structure and/or converting large protein aggregates into smaller species [5,6,12,13]. Aggregates of reduced size, resulting from a ClpB-dependent fragmentation process, may then serve as substrates for KJE, which has

a disaggregation activity towards small protein aggregates [5,14,15]. This "crowbar" activity of ClpB has been proposed to be mediated by the M-domain, which is unique to ClpB homologs. Consistent with this model, the M-domain is essential for ClpB-mediated protein disaggregation [16–18]. The recently solved crystal structure of *Thermus thermophilus* ClpB revealed that the M-domain is forming a large coiled-coil structure that is located on the outer surface of a hexameric ClpB model [12]. Interestingly, the M-domain is flexible and its mobility is crucial for protein disaggregation [12].

An alternative mechanism for protein disaggregation by ClpB involves the continuous extraction of unfolded proteins from the aggregate by ClpB/KJE. Such a process could involve a translocation activity of ClpB, which was recently suggested to be an integral part of the disaggregation reaction [19–21]. This potential activity of ClpB would be reminiscent of the disaggregation activity of peptidase-associated AAA+ proteins that can degrade aggregated proteins. The *E. coli* proteolytic system ClpA/ClpP hydrolyzes aggregated MDH, an activity that is further increased in the presence of the ClpA-specific adaptor protein ClpS [22]. However, ClpA/ClpP cannot replace ClpB/KJE function in vivo, and therefore it is unclear whether such a mechanism is also the basis for ClpB-dependent protein disaggregation [9,22].

Using aggregated MDH as a model system, a clear distinction between the two models has so far been prevented, because the solubilization of MDH aggregates and the subsequent MDH refolding reaction were not tightly coupled. Thus, initial enzymatic activity of refolded MDH was only observed after a lag phase when the disaggregation reaction was almost finished [6]. This observation might be explained by ClpB-dependent aggregate fragmentation first, followed by further solubilization and substrate refolding by KJE, however, evidence for such a process is missing.

Here, we reinvestigated the ClpB/KJE-mediated disaggregation of MDH aggregates in more detail, by determining changes in two characteristic parameters of the aggregates during the disaggregation reaction: turbidity and solubility. Changes in turbidity and solubility of MDH aggregates were tightly coupled and occurred with comparable kinetics. Soluble MDH species, isolated at various time points of the disaggregation reaction, predominantly consisted of MDH monomers and dimers, indicating that the solubilization of MDH aggregates is mediated by the continuous extraction of unfolded MDH molecules from the aggregate.

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2. Materials and methods

2.1. Proteins

ClpB was purified as described after overproduction in Δ*clpB::kan* cells [16]. Purifications of DnaK, DnaJ, GrpE, GroEL, and GroES were performed as described previously [6,9]. α-glucosidase and Pyruvate kinase were purchased from Sigma and pig heart muscle MDH from Roche. Protein concentrations were determined with the Bio-Rad Bradford assay using BSA as standard. Protein concentrations refer to the protomer.

2.2. In vitro disaggregation assays

MDH (2 μM) was denatured at 47 °C for 30 min in buffer A (50 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgCl$_2$, and 2 mM DTT). α-Glucosidase (1 μM) was denatured in buffer A at 50 °C for 45 min. Protein disaggregation and refolding were started by incubating aggregated proteins and chaperones (KJE: 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE; ELS: 2 μM GroEL, 2 μM GroES; 1.5 μM ClpB; and GroEL trap: 7 μM GroEL D87K) in buffer A at 30 °C. All assays were performed in the presence of an ATP-regenerating system (3 mM phosphoenol pyruvate; 20 μg/ml pyruvate kinase; and 2 mM ATP). Turbidity of protein aggregates was measured at an excitation and emission wavelength of 550 nm (Perkin–Elmer luminescence spectrometer LS50B). Disaggregation rates were derived from the linear decrease of aggregate turbidity. Determination of enzymatic activities followed published protocols [6,23]. Refolding rates were calculated from the linear increase of substrate activities.

2.3. Analysis of soluble MDH species

Labelling of MDH was performed by use of *N*-Succinimidyl [2,3-^{3}H] propionate (Amersham) as described [24]. Solubilized ^{3}H-MDH was isolated at different time points of the disaggregation reaction by centrifugation (13 000 rpm, 30 min, 4 °C) after inhibiting the disaggregation reaction by addition of 100 mM EDTA and 100 mM (NH$_4$)$_2$SO$_4$. Soluble ^{3}H-MDH was quantified by scintillation counting. ^{3}H-labelled MDH species, occurring during the disaggregation reaction, were separated by S200 HR10-30 (Amersham) size exclusion chromatography and quantified by scintillation counting. Size exclusion chromatography was performed at 4 °C in buffer A containing 5% (v/v) glycerol.

2.4. Dynamic light scattering measurements

For dynamic light scattering measurements, 2 μM MDH was denatured in buffer A at 47 °C for 3.5 min in a quartz cuvette inside a DynaPro-MS/X instrument (Protein Solutions Ltd., High Wycombe, Bucks., UK). The reaction was cooled to 30 °C and chaperones (KJE: 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE; 1.5 μM ClpB) were added subsequently. Particle sizes were calculated by the software DYNAMICS (version 6.0) supplied by the manufacturer.

3. Results and discussion

3.1. Protein disaggregation relies on the continuous extraction of unfolded molecules

We followed the changes in aggregate turbidity as well as substrate solubility and activity during the disaggregation of heat aggregated MDH. In order to allow a precise calculation of still aggregated versus resolubilized substrate, MDH was labelled with ^{3}H. Labelled MDH was fully active and exhibited the same aggregation and disaggregation kinetics as non-labelled MDH (data not shown). ^{3}H-MDH aggregates, generated by 47 °C heat treatment for 30 min, appeared stable for hours as determined by centrifugation and did not release soluble species in the presence of KJE or ClpB alone (Table 1). The increase in soluble MDH species, generated by ClpB/ KJE, occurred with the same kinetics as the decrease in MDH aggregate turbidity. In contrast the MDH activity, measured simultaneously, showed a lag phase of 10–20 min and

Table 1
Solubilization and refolding of MDH from aggregates

Chaperones	Disaggregation rate (% turbidity/min)	Solubilization rate (% soluble MDH species/min)	Refolding rate (% refolded MDH/min)
No chaperones	0	0	0
KJE/ClpB	3.09	2.7	0.73
KJE/ClpB/ESL	3.0	2.9	2.95
KJE/ClpB/GroEL-Trap	3.09	2.78	0.06
KJE	0.004	0.007	0.005
ClpB	0	0	0
ESL	0	0	0
GroEL-Trap	0	0	0
KJE/ESL	0.004	0.008	0.01
ClpB/ESL	0	0	0
KJE/GroEL-Trap	0.003	0.008	0
ClpB/GroEL-Trap	0	0	0

MDH (2 μM) was heat denatured as described in the experimental procedures. MDH disaggregation was initiated at 30 °C upon dilution of the substrate (1:1) with the indicated chaperones. Disaggregation, solubilization and refolding rates were determined as described in Section 2.

proceeded with a rate that was 4-fold reduced compared to that determined for MDH disaggregation and solubilization (Fig. 1A; Table 1). As a consequence, disaggregation and solubilization of MDH aggregates were completed after 60 min, whereas complete MDH refolding required 2–3 h.

Solubilized MDH species may represent either small soluble MDH aggregates, generated by the fragmentation of large aggregates, or single extracted unfolded MDH molecules, or a mixture of both forms. We therefore determined the nature of solubilized MDH species by subjecting the soluble fractions, isolated at different time points of the disaggregation reaction, to size exclusion chromatography analysis. At all time points of the solubilization process only monomeric and dimeric MDH, but no larger soluble MDH species, were observed. Since injected, ^{3}H-MDH species were recovered with more than 80% yield in all runs, monomeric and dimeric MDH represent the predominant species that are extracted from the aggregates by ClpB/KJE (Fig. 1B). Dimeric MDH species reflect native MDH, but potentially also still inactive molecules, since dimeric MDH was already detectable after 10 min without exhibiting enzymatic activity. Monomeric MDH species were 1.8-times more populated than MDH dimers at the beginning of the disaggregation reaction. With time the ratio between MDH monomers and dimers decreased, reflecting folding of the extracted MDH molecules to the active, dimeric form (Fig. 1B, inset). After incubation for 120 min, the refolding of aggregated MDH was largely complete and, consistently, only dimeric ^{3}H-MDH species were recovered.

3.2. Analysis of MDH disaggregation by dynamic light scattering

Extraction of monomeric and dimeric MDH molecules by ClpB/KJE should result in a continuous decrease of MDH aggregate size. Changes in aggregate size during the disaggregation reaction were directly followed by dynamic light scattering measurements. Size determination by this method relies on globular particle shape, which was confirmed for MDH aggregates by atomic force microscopy (data not shown). It should be noted that this set of experiments required a shorter dena-
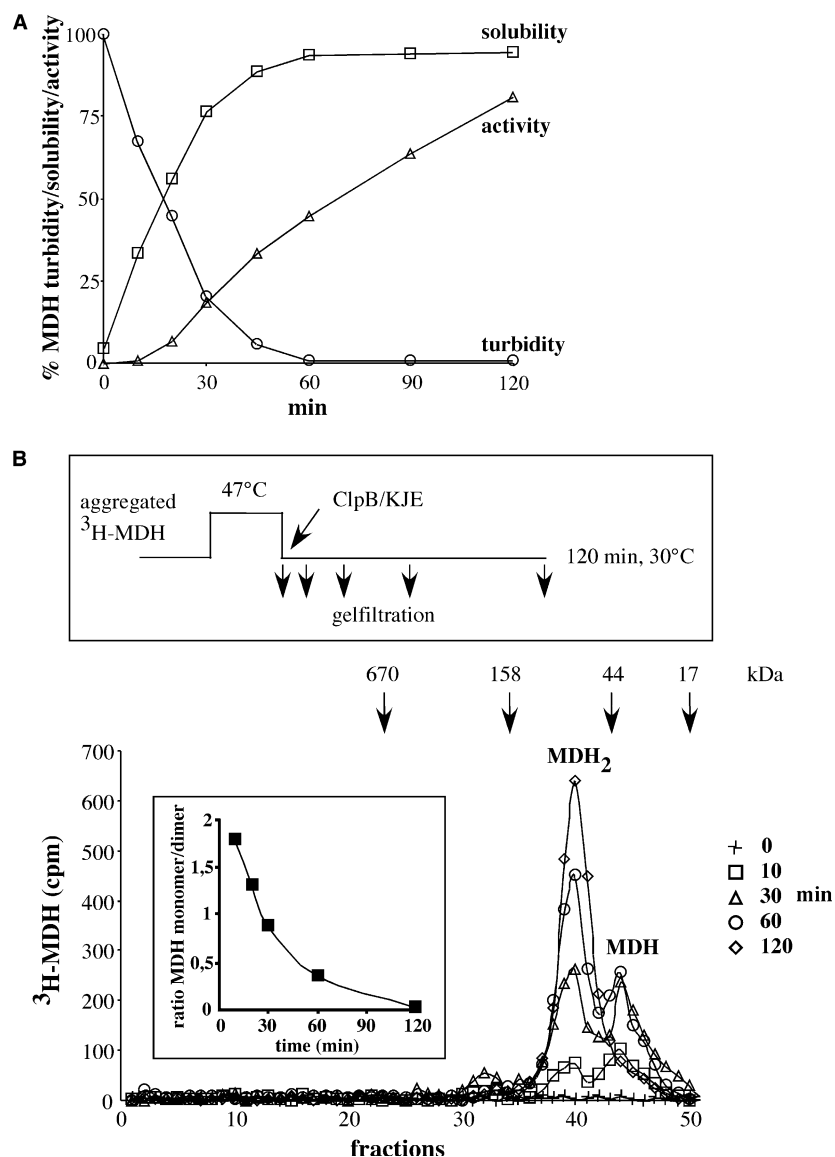
Fig. 1. Protein disaggregation relies on the continuous extraction of unfolded molecules. (A) Changes in characteristic parameters of MDH aggregates during the ClpB/KJE-mediated disaggregation reaction. Disaggregation of aggregated MDH by KJE and ClpB was followed at 30 °C by determining changes in aggregate turbidity, MDH solubility and activity. Turbidity of MDH aggregates was set at 100%. Solubility and enzymatic activity of native MDH were set at 100%. (B) Soluble ^{3}H-MDH species, isolated at different time points of the disaggregation reaction (as in A), were analyzed by size exclusion chromatography and quantified by scintillation counting. A protein standard is given.

turation period of MDH to ensure light scattering measurements within the detection range of the instrument. Short incubation of MDH at 47 °C caused the formation of stable protein aggregates with an average radius of 33 nm (Fig. 2A). The generated aggregates were quite homogenous, exhibiting a polydispersity of 23%, corresponding to an aggregate size range from 20 to 50 nm.

In the presence of ClpB and KJE, a continuous decrease of the MDH aggregate size was observed (Fig. 2A). After 3 min incubation time, the measurement was dominated by light scattering of hexameric ClpB and, as a consequence, no further decrease in particle size was noticed. Addition of ClpB (+ATP) alone did neither affect the average size nor the size distribution of aggregated MDH and smaller MDH aggregates were not detected (Fig. 2B). Instead, an increase in light scattering was observed when the aggregates were incubated with KJE

or ClpB (+ATPγS), indicating the formation of larger MDH particles (Fig. 2B). This increase in aggregate size could be attributed to the association of DnaK or ClpB with MDH aggregates: binding of ClpB or DnaK/DnaJ to aggregated MDH was demonstrated by their co-aggregation upon centrifugation, whereas incubation with native MDH resulted only in background levels of chaperones in the pellet fraction (Fig. 2C).

3.3. GroEL stimulates the refolding of extracted, unfolded MDH, without influencing MDH disaggregation

Since soluble MDH species appeared with a faster kinetic than active MDH molecules during the disaggregation reaction, refolding of already extracted MDH by KJE seems to be the rate-limiting step that causes the initially observed
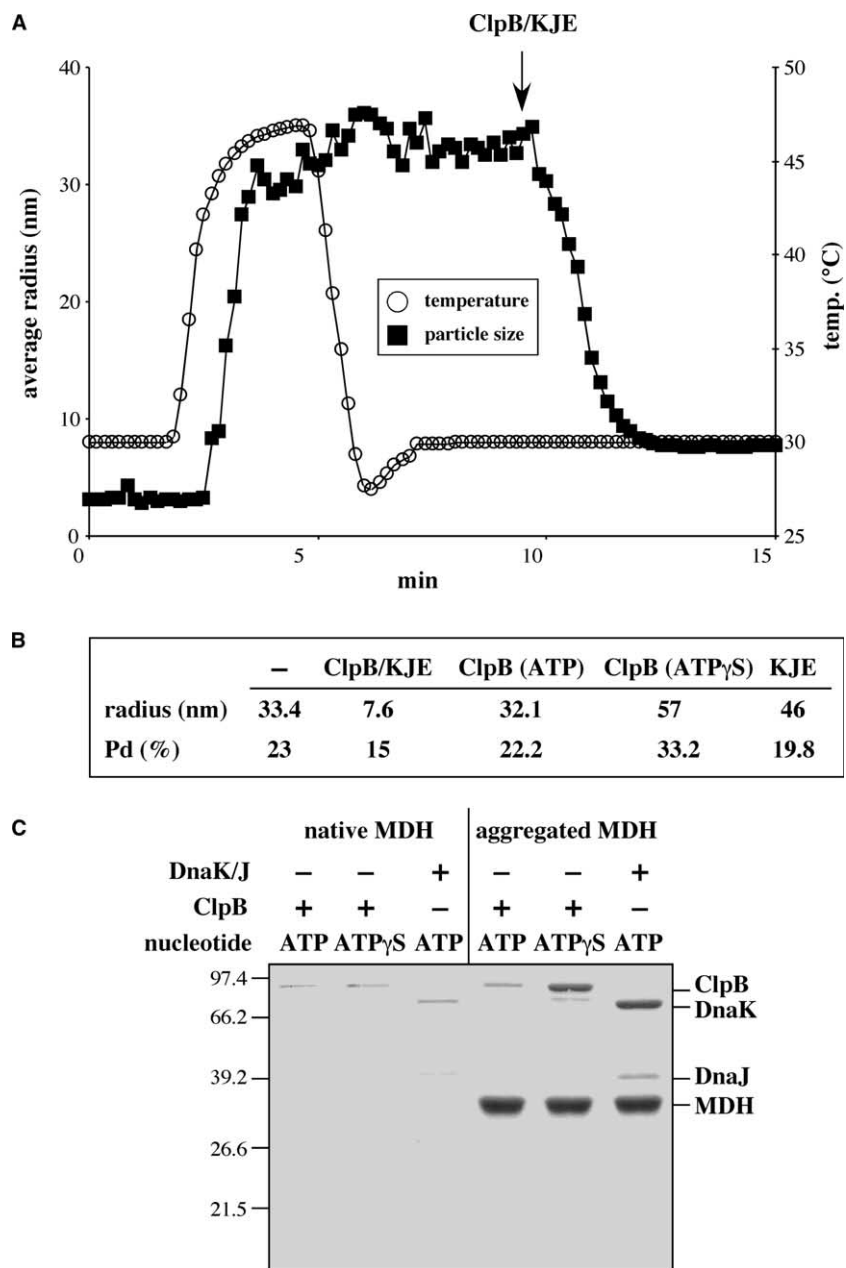
Fig. 2. MDH aggregation and disaggregation analyzed by dynamic light scattering. (A) 2 μM MDH was denatured at 47 °C and aggregation was followed by determining changes of MDH size. Aggregated MDH was subsequently incubated at 30 °C in the presence of ClpB/KJE and changes in the aggregate size were calculated. (B) MDH aggregates were incubated in the absence (−) or presence of chaperones as indicated for 10 min at 30 °C. Calculated average particle size (radius) and polydispersity (Pd) are given. (C) Chaperones were incubated with either native or aggregated MDH for 10 min at 30 °C. MDH aggregates were isolated by centrifugation (45.000 rpm, 30 min, 4 °C) and chaperone association was analyzed by Coomassie-stained SDS–PAGE.

discrepancies between the rates of MDH disaggregation and refolding. Unfolded MDH has been shown to be fast and efficiently refolded by the GroEL chaperone and its co-chaperone GroES (ELS) [25]. We followed the ClpB/KJE-dependent reactivation of aggregated MDH in the additional presence of ELS or a GroEL mutant derivative (EL-D87K) that binds but does not release substrates [26]. While ELS alone did not support MDH reactivation from aggregates, it increased the MDH refolding rate 4-fold in the presence of ClpB/KJE (Fig. 3A, Table 1). The observed MDH refolding rate in the presence of ClpB/KJE/ELS (2.95% refolded MDH/min) was comparable

to the disaggregation rate of MDH aggregates by ClpB/KJE only (3.09% turbidity/min) and, consistently, a lag phase for MDH reactivation was no longer observed. Importantly, addition of ELS did neither influence the ClpB/KJE-dependent disaggregation reaction (decrease of turbidity), nor the occurrence of soluble MDH species, demonstrating that ELS activity was restricted to the refolding of non-native, soluble MDH after its extraction from aggregates by ClpB/KJE (Fig. 3A, Table 1). Consistent with these findings, the GroEL trap variant (EL-D87K) inhibited only MDH refolding without affecting the solubilization process of MDH aggregates (Fig.
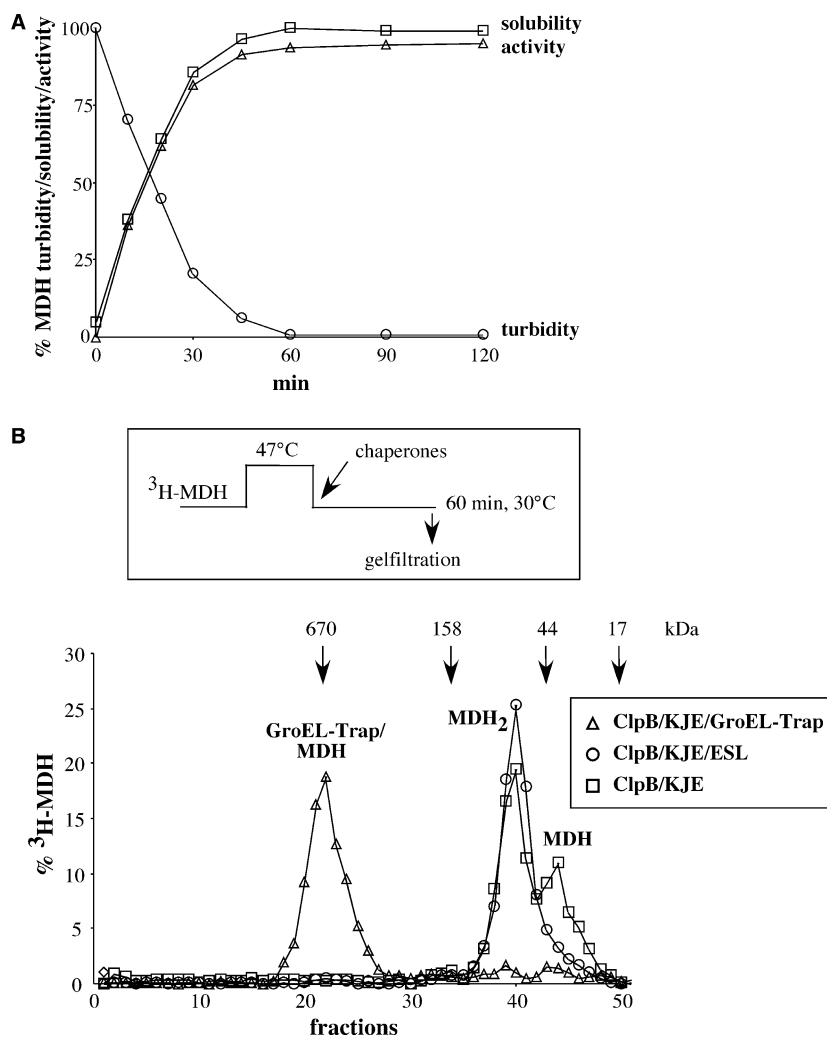
Fig. 3. GroELS speeds up the refolding of extracted MDH molecules. (A) Disaggregation of 1 μM aggregated MDH by ClpB/KJE was followed in the additional presence of ELS. Changes in aggregate turbidity, MDH solubility and activity were determined. (B) Soluble ^{3}H-MDH species, isolated 60 min after addition of the indicated chaperones, were analyzed by gel filtration and quantified by scintillation counting. The amount of ^{3}H-MDH was set at 100%. A protein standard is given.

3A, Table 1). Analysis of ^{3}H-MDH-species, generated during the disaggregation reaction in the additional presence of EL-D87K, revealed that the vast majority of released MDH molecules was bound to the GroEL mutant, explaining the inhibition of MDH refolding (Fig. 3B). In contrast, addition of ELS to ClpB/KJE led to the formation of native, dimeric MDH species whereas MDH monomers were no longer detectable (Fig. 3B). These data demonstrate that the previously reported differences between the disaggregation and refolding reactions of aggregated MDH are caused only by slow KJE-mediated substrate refolding, but not by an initial ClpB-mediated fragmentation process.

3.4. Direct coupling of disaggregation and refolding of another aggregated model substrate

To address the general nature of our findings, we followed the disaggregation and the subsequent refolding reactions of heat-aggregated α-glucosidase as an alternative substrate (Fig. 4). The decrease in aggregate turbidity and the increase in enzymatic activity were tightly coupled and no lag phase in substrate refolding was observed. Protein disaggregation and substrate refolding occurred with similar rates, suggesting

that solubilization of aggregated α-glucosidase follows the same process as demonstrated for MDH aggregates in this work.
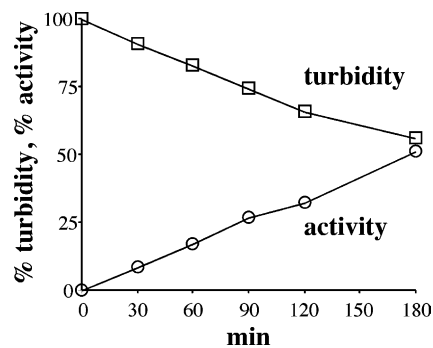

Fig. 4. Direct coupling of disaggregation and refolding reactions of heat-aggregated α-glucosidase. Heat-aggregated α-glucosidase (1 μM) was incubated in the presence of ClpB (1 μM) and KJE (1 μM DnaK, 0.2 μM DnaJ, and 0.1 μM GrpE). Protein disaggregation was followed by determining the decrease in aggregate turbidity and increase in enzymatic activity. The turbidity of α-glucosidase aggregates and the activity of native α-glucosidase were set at 100%.

Taken together, our data indicate that protein disaggregation by ClpB/KJE is mediated by the continuous extraction of unfolded proteins from the aggregate. Upon solubilization, the unfolded polypeptides can be subsequently refolded by a chaperone network independent of ClpB. The previously noticed discrepancies between ClpB/KJE-dependent MDH disaggregation and refolding are caused by slow KJE-mediated refolding of solubilized unfolded MDH molecules and are restricted to this model protein.

The continuous one-by-one removal of unfolded proteins from aggregates can be explained by ClpB-dependent threading of substrates through the central pore of the AAA+ oligomer. Such a mechanism is supported by studies on ClpB/Hsp104 pore mutants, which are affected in protein disaggregation [19,20]. Indeed, direct evidence for a threading mechanism was obtained very recently [27]. We did not gain evidence for a ClpB-dependent fragmentation process as basis for aggregate solubilization. The postulated crowbar activity of the M-domain may therefore play a different role, e.g., by facilitating the extraction process of unfolded substrates that are trapped within the aggregate. Interestingly, a fragmentation activity of Hsp104 towards Sup35-NM fibers was recently suggested [28,29]. Since ClpB did not exhibit such an activity in the same studies, it is possible that this mode of Hsp104 action is restricted to this special substrate. Further studies are required to clarify the potential mechanistic differences between disaggregation of heat-aggregated proteins and amyloid fibrils.

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