The Amino-terminal Domain of ClpB Supports Binding to Strongly Aggregated Proteins*

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Micheal E. Barnett, Maria Nagy, Sabina Kedzierska¹, and Michal Zolkiewski²

From the Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506

Bacterial heat-shock proteins, ClpB and DnaK form a bichaperone system that efficiently reactivates aggregated proteins. ClpB undergoes nucleotide-dependent self-association and forms ring-shaped oligomers. The ClpB-assisted dissociation of protein aggregates is linked to translocation of substrates through the central channel in the oligomeric ClpB. Events preceding the translocation step, such as recognition of aggregates by ClpB, have not yet been explored, and the location of the aggregate-binding site in ClpB has been under discussion. We investigated the reactivation of aggregated glucose-6-phosphate dehydrogenase (G6PDH) by ClpB and its N-terminally truncated variant ClpBΔN in the presence of DnaK, DnaJ, and GrpE. We found that the chaperone activity of ClpBΔN becomes significantly lower than that of the full-length ClpB as the size of G6PDH aggregates increases. Using a “substrate trap” variant of ClpB with mutations of Walker B motifs in both ATP-binding modules (E279Q/E678Q), we demonstrated that ClpBΔN binds to G6PDH aggregates with a significantly lower affinity than the full-length ClpB. Moreover, we identified two conserved acidic residues at the surface of the N-terminal domain of ClpB that support binding to G6PDH aggregates. Those N-terminal residues (Asp-103, Glu-109) contribute as much substrate-binding capability to ClpB as the conserved Tyr located at the entrance to the ClpB channel. In summary, we provided evidence for an essential role of the N-terminal domain of ClpB in recognition and binding strongly aggregated proteins.

Unlike conventional molecular chaperones, which prevent but do not reverse protein aggregation, bacterial ClpB in cooperation with DnaK, DnaJ, and GrpE efficiently reactivates strongly aggregated proteins (1–3). Similar bichaperone systems involving ClpB and DnaK homologues have been also found in yeast (4) and plants (5). ClpB belongs to the AAA+ superfamily of ATPases associated with various cellular activities (6). ClpB contains a distinct N-terminal domain loosely associated with the rest of the protein (7) and two AAA+ ATP-binding modules separated by a coiled-coil domain (8). Like other AAA+ ATPases, ClpB forms ring-shaped hexamers in the presence of nucleotides (9, 10).

Recently, it has been shown that the mechanism of protein disaggregation mediated by ClpB involves ATP-dependent threading of substrates through the central channel in the oligomeric ring (11), which implies that ClpB binds exposed termini of aggregated polypeptides. Thus, the mechanism of ClpB is analogous to that of ClpA, ClpX, HslU (ClpY), and a number of eukaryotic AAA+ ATPases that translocate and unfold substrates and deliver them to the associated peptidases for degradation (12). The crucial remaining questions concern the role of DnaK/DnaJ/GrpE in the ClpB-assisted substrate unfolding/reactivation and the mechanism of substrate recognition by ClpB.

Because aggregated proteins, unlike degradation substrates, are not tagged, ClpB should possess unique aggregate-recognition capabilities that are distinct from those of ClpA, ClpX, and HslU. A protein machine that is capable of unfolding any polypeptide whose termini are exposed would be harmful rather than beneficial in vivo. Studies on peptide binding to ClpB revealed preferential interactions with positively charged and, to a lower extent, with aromatic residues (13). A tyrosine and a pair of acidic residues located at the N-terminal entrance to the ClpB channel are involved in substrate binding (13). However, that group of amino acids is located within the AAA+ module (between Walker A and Walker B motifs) and is found in many AAA+ ATPases, regardless of their function (13). This suggests that those conserved residues may play a role in initiating the ratcheting mechanism of substrate threading rather than in substrate recognition.

We hypothesized that the N-terminal domain of ClpB may contain sequence or structural motifs that are essential for the capability of ClpB to recognize and bind aggregated proteins. Sequence similarity between the N-terminal regions of ClpB and other AAA+ ATPases is low and limited to a few short amino acid motifs (14). The role of the N-terminal domain of ClpB is unknown and has been under an intense discussion. Importantly, the ClpB transcript contains an alternative translation initiation site at the N terminus of the first AAA+ module. Expression of the ClpB gene results in production of the full-length 95-kDa ClpB as well as the truncated 80-kDa ClpBΔN (15, 16). Thus, investigating the function of the N-terminal domain may also help explain the physiological role of the ClpB isofrom lacking that domain.

Published data suggest that the N-terminal domain plays a role in the reactivation of aggregated substrates (17). Mutations of selected residues within the N-terminal domain of ClpB inhibit its chaperone activity and casein-induced activation of ClpB ATPase (14, 18). The isolated N-terminal domain of ClpB binds to misfolded proteins and displays “chaperone-like” aggregation-suppressing properties (19). In contrast, other studies implied that the N-terminal domain is not essential for the function of ClpB. Both the full-length ClpB and ClpBΔN contributed to the survival of bacteria during heat shock (20). Some aggregated substrates could be reactivated in vitro by the bichaperone system containing either ClpB or ClpBΔN (21). Thus, the N-terminal domain does not appear to play a direct role in the mechanism of substrate threading, which is mediated by the channel-forming domains of the AAA + modules. Finally, mutant substrate-trap variants of ClpB and ClpBΔN interacted with selected protein substrates to a similar extent (22).

In summary, the role of the N-terminal domain of ClpB remains controversial. In this study, we tested the hypothesis that the above discrepancy among results from different assays comparing the full-length ClpB

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1 Present address: Dept. of Biochemistry, University of Gdansk, Gdansk, Poland.

2 To whom correspondence should be addressed: Dept. of Biochemistry, Kansas State University, 104 Willard Hall, Manhattan, KS 66506. Fax: 785-532-7278; E-mail: michaelz@ksu.edu.
with ClpBΔN arises from differences in properties of aggregates presented to ClpB as potential substrates. We examined the chaperone activity and aggregate-binding capability of ClpB and ClpBΔN using a substrate with a controllable extent of aggregation. We found that the N-terminal domain of ClpB significantly contributes to the aggregate-binding affinity and becomes particularly essential for binding large protein aggregates.

**EXPERIMENTAL PROCEDURES**

*Proteins—* Previously published procedures were used to produce and purify the Escherichia coli chaperones, ClpB and ClpBΔN (17), DnaK (23), and DnaJ (24). GrpE was obtained from StressGen Biotechnologies (Victoria, Canada). Site-directed mutagenesis of ClpB was performed using the QuikChange method (Stratagen), and the mutated ClpB variants were purified as described for wild type ClpB (17). Glucose-6-phosphate dehydrogenase (54 kDa, monomer) (G6PDH)3 from Leuconostoc mesenteroides was obtained from Sigma. Protein concentrations were determined spectrophotometrically and are given in monomer units.

**G6PDH Aggregate Production**—To prepare aggregated G6PDH, 5 μl of the 600 μM stock solution was mixed with an equal volume of the heated denaturation buffer (10 mM urea, 16% glycerol, and 40 mM dithiothreitol). The mixture was incubated at 47 °C for 5 min, at which time 90 μl of the refolding buffer (50 mM triethanolamine/Cl, pH 7.5, 20 mM Mg(OAc)2, 30 mM KCl, 1 mM β-mercaptoethanol, and 1 mM EDTA) without or with 2 mM ATP or ADP was added, and the G6PDH sample was mixed vigorously and incubated at 47 °C for a variable period of time. The solution was then mixed briefly and incubated on ice for 2 min followed by centrifugation at 4 °C for 10 min at 13,000 × g.

**Gel-filtration Chromatography**—Soluble G6PDH aggregates were chromatographed with or without ClpB or its variants. Protein samples were injected onto a 25-cm Pharmacia HR10/30 column packed with Superose 6 gel-filtration medium (Amersham Biosciences) equilibrated with the refolding buffer with 2 mM ATP or ADP or without nucleotides. Elution at 0.5 ml/min was performed at room temperature using a Shimadzu HPLC LC10ATvp equipped with a SPD-M10Avp diode-array detector. Gel-filtration standards were from Bio-Rad.

One-minute fractions (0.5 ml) were collected from the column and analyzed by SDS-PAGE followed by Coomassie staining or Western blotting. Some eluted fractions were concentrated by precipitation with ice-cold acetone was added to the pellet. The samples were again centrifuged for 30 min under the same conditions. After removing the acetone, 20 μl of 5× SDS loading buffer was added to the pellet, the sample was boiled for 2 min, and analyzed by SDS-PAGE. For Western blotting, the samples were transferred to nitrocellulose membrane and incubated with rabbit polyclonal anti-ClpB antibody (7) and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL). Blots were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**G6PDH Reactivation Assay**—Aggregates of G6PDH (22 μM) were diluted 8-fold into the refolding buffer with 5 mM ATP containing no chaperones, 1 μM DnaK with 0.5 μM DnaJ and 0.5 μM GrpE (KJE), KJE with 1.5 μM ClpB, or KJE with 1.5 μM ClpBΔN. After incubation at 30 °C, aliquots were withdrawn, and the G6PDH activity was measured.

**G6PDH Activity Assay**—G6PDH samples were incubated at 30 °C in 50 mM Tris-Cl, pH 7.8, 5 mM MgCl2 with 2 mM glucose-6-phosphate and 1 mM NADP+. The absorption at 340 nm was measured after 10 min.

**ClpB ATPase Assay**—ClpB samples were incubated at 37 °C in 100 mM Tris-HCl, pH 8, 10 mM MgCl2, 5 mM ATP, 1 mM EDTA, 1 mM dithiothreitol with 0.1 mg/ml κ-casein (Sigma). Inorganic phosphate production was determined using the malachite green method (25, 26).

**RESULTS**

We produced aggregates of G6PDH using a procedure analogous to that developed by Goloubinoff and co-workers (27). Native G6PDH was first unfolded in urea at 47 °C and then rapidly diluted into a refolding buffer and further incubated at 47 °C. As has been shown before (27), G6PDH does not spontaneously refold under such conditions, instead it misfolds and starts to aggregate. The aggregation process can be effectively arrested at different stages by rapid cooling of the protein samples. The resulting G6PDH solutions exhibited variable turbidity, but their soluble fractions showed consistent elution patterns in gel-filtration chromatography. As shown in Fig. 1, the apparent size of the aggregates depends on the time of refolding at 47 °C. Immediately after the initiation of refolding, G6PDH eluted with an apparent size of ~200 kDa, which is consistent both with the expanded size of unfolded monomeric G6PDH and with formation of small aggregates. After 1 min of refolding, the elution pattern showed a broad distribution of particles with a maximum at ~1,000 kDa. After 15 min of refolding, the majority of G6PDH eluted in the void volume fraction corresponding to ~2,000 kDa with a minor fraction of smaller aggregates.

Goloubinoff and co-workers (27) concluded that small aggregates of G6PDH could be reactivated by the DnaK/DnaJ/GrpE system alone, whereas the reactivation of large aggregates required ClpB. We compared the rates of G6PDH reactivation by different chaperones for the aggregate population prepared with 1- and 15-min refolding time (see Fig. 1). As shown in Fig. 2A (open circles), the 1-min aggregate population showed measurable G6PDH activity that did not increase over time, which indicated that the aggregate population contains a fraction of refolded G6PDH. The amount of refolded G6PDH did not increase spontaneously, which is consistent with the irreversible nature of protein aggregation. Either DnaK/DnaJ/GrpE alone or in cooperation with ClpB reactivated aggregated G6PDH, but the reactivation rate was

![Image](http://www.jbc.org/)

**G6PDH Activity**

**Gel-filtration analysis of aggregated G6PDH.** Urea-denatured 300 μM G6PDH was diluted 10-fold into the refolding buffer at 47 °C. The samples were analyzed immediately (solid line), after a 1-min incubation at 47 °C and 2 min on ice (dotted line), or after a 15-min incubation at 47 °C and 2 min on ice (broken line) on a Superose 6 column equilibrated with the refolding buffer with 2 mM ATP at 0.5 ml/min. Filled circles correspond to the elution times of blue dextran (2 MDa), thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

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3 The abbreviation used is: G6PDH, glucose-6-phosphate dehydrogenase.
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~4-fold higher when ClpB was present. The rate of G6PDH reactivation was ~2-fold lower when the full-length ClpB was replaced with ClpBΔN.

In contrast to the 1-min aggregate sample, the 15-min aggregate population did not show G6PDH activity (Fig. 2B, open circles), which indicates that essentially all G6PDH aggregated during 15 min of refolding. The DnaK/DnaJ/GrpE system alone did not reactivate G6PDH, which is consistent with previous observations (27). Either ClpB or ClpBΔN reactivated G6PDH, but the reactivation rate in this case was ~5-fold lower with ClpBΔN than with the full-length ClpB. Collectively, the results in Fig. 2 indicate that the chaperone activity of ClpBΔN is weaker than that of ClpB. The ClpBΔN deficiency in processing aggregated G6PDH is exacerbated when the effective size of the aggregates increases (compare Figs. 1, 2A, and 2B).

To further investigate the reasons for the ClpBΔN deficiency in reactivating large aggregates, we compared the amounts of ClpB and ClpBΔN bound to the aggregates. Large G6PDH aggregates were prepared after the 15-min refolding, incubated with ClpB or ClpBΔN in the presence of ATP with the ratio [G6PDH]/[ClpB] as in Fig. 2, and analyzed with gel-filtration chromatography. Without G6PDH, ClpB and ClpBΔN eluted as hexamers with an apparent molecular weight ~600 kDa and did not contain large aggregated particles (Fig. 3, A, B, and D).

In the presence of aggregated G6PDH, a small amount of ClpB, but not ClpBΔN was detected in the aggregate fractions after trichloroacetic acid precipitation (Fig. 3, C and E). This result indicates that the lower G6PDH reactivation rate of ClpBΔN versus ClpB might be because of a lower substrate-binding affinity.

Because AAA+ ATPases interact with their substrates in the ATP-bound state (28), the amount of ClpB or ClpBΔN found in gel-filtration fractions containing aggregated G6PDH may be affected by either substrate-binding affinity or the lifetime of the ATP-bound conformation of the ATPase. To observe the interactions between ClpB or ClpBΔN and the aggregates in the absence of ATP hydrolysis, we employed ATPase-deficient mutants of ClpB. Weibezahn et al. (22) described a substrate-trap variant of ClpB with mutations of Walker B motifs in both AAA+ modules (E279A/E678A). We produced analogous variants of ClpB and ClpBΔN with the mutations of essential Walker B glutamates (E279Q/E678Q). We also produced a ClpB variant with mutations of sensor-1 motifs in both AAA+ modules (T315A/N719A). It has been postulated that hydrogen-bonding residues of sensor-1 play a role in synchronization of ATP hydrolysis in all catalytic sites within the AAA+-ring (29), and their mutations might, therefore, inhibit the substrate threading reaction. Indeed, single sensor-1 mutations have been found to inhibit the ATPase of yeast Hsp104 (30).
Both the double Walker B and double sensor-1 mutants of ClpB formed ATP-dependent oligomers, similar to wild type ClpB and ClpBΔN (data not shown). However, neither mutant rescued the growth of the clpB-null strain of E. coli at 50 °C (data not shown), which indicates that mutations of conserved motifs in both AAA+ modules strongly inhibit the chaperone activity of ClpB. As shown in Fig. 4A, the sensor-1 mutations inhibited partially, and the Walker B mutations completely, the ATPase of ClpB in the presence of casein, an activator of ATPase and a substrate analog. The amount of the double sensor-1 mutations inhibited partially, and the Walker B mutations strongly inhibit the chaperone activity of ClpB. As shown in Fig. 4E, indicates that mutations of conserved motifs in both AAA modules completely, the ATPase of ClpB in the presence of casein, an activator of ATPase and a substrate analog. The amount of the double sensor-1 mutations inhibited partially, and the Walker B mutations strongly inhibit the chaperone activity of ClpB.

Next, we asked whether trap ClpBΔN would preferentially interact with a specific size of G6PDH aggregate. We prepared a broad distribution of aggregates by using a 1-min refolding time (see Fig. 1). As shown in Figs. 6, B and D, the binding of trap ClpBΔN to G6PDH aggregates not much larger than ClpB itself is evident for the elution at 19 and 20 min. However, the amounts of trap ClpBΔN decrease significantly for earlier elution times that correspond to larger G6PDH aggregates. The full-
Substrate Binding to ClpB

FIGURE 7. Binding of ClpB traps with the N-terminal and channel mutations to strongly aggregated G6PDH. A, large aggregates of G6PDH (25 μM) were prepared as in Fig. 5 and incubated with 15 μM E279Q/E678Q, (E279Q/E678Q)ΔN, D103A/E109A/E279Q/E678Q, or Y251A/E279Q/E678Q. Gel-filtration fractions eluted at 11 and 12 min (see Fig. 3) were concentrated by trichloroacetic acid precipitation, and analyzed by SDS-PAGE followed by Coomassie staining. B, band densitometry of ClpB variants shown in A. Shown are the totals of fractions 11 and 12 for each protein sample.

FIGURE 8. Location of conserved acidic residues in the N-terminal domain of ClpB. Shown is the monomer structure of ClpB from Thermus thermophilus (8). The positions of flexible diaphragms along the ClpB channel are indicated by their flanking residues: Glu-246 and Gln-651 (purple). Asp-103 is shown in orange, and Glu-109 is shown in red.

length trap ClpB binds with a similar efficiency to all but the largest G6PDH aggregates (Fig. 6, A and E). The binding affinity of trap ClpBΔN is lower than that of the full-length ClpB for all aggregate sizes, and it is at least an order of magnitude lower for very large aggregates (Figs. 6E and 7). We concluded that whereas some aggregate-binding site(s) do reside in ClpBΔN, presumably at the channel entrance (13), the N-terminal domain of ClpB contributes significantly to the aggregate-binding capability of the chaperone and supports binding to both small and large aggregates.

We have shown earlier that the conserved residues within the N-terminal domain of ClpB, Thr-7, Asp-103, and Glu-109, support the chaperone activity (14). We asked whether the interactions of trap ClpB with aggregated G6PDH are affected by mutations in the N-terminal domain. Because positively charged peptides bind preferentially to ClpB (14), we focused on the pair of acidic residues and produced a quadruple mutant D103A/E109A/E279Q/E678Q, i.e. the ClpB trap with mutations of the Asp/Glu pair in the N-terminal domain. As has been shown before, the mutations in the N-terminal domain do not destabilize its structure nor do they affect the oligomerization and the basal ATPase activity of ClpB (14). We also produced the ClpB trap variant with the mutation of the conserved Tyr at the entrance to the channel: Y251A/E279Q/E678Q. As shown in Fig. 7, both the N-terminal mutant and the channel mutant bind less efficiently to large aggregates of G6PDH than the full-length ClpB, but more efficiently than ClpBΔN. We concluded that the pair of acidic residues in the N-terminal domain of ClpB contributes significantly to the aggregate-binding affinity.

DISCUSSION

It is believed that the multitude of functions performed by AAA+ ATPases is achieved by a variability of substrate-recognition domains covalently attached to the energy-transducing AAA+ modules (6). In the case of ClpB, its N-terminal domain forms an independently folded structural unit that is attached to the first AAA+ module with a flexible linker (8). Thus, the N-terminal domain of ClpB is a candidate for the substrate-sensing “attachment” domain, but its role in substrate binding has not been demonstrated yet. In this work, we have shown directly that the binding of ClpB to protein aggregates is supported to a high extent by molecular contacts provided by the N-terminal domain. We have also identified two residues within the N-terminal domain, Asp-103 and Glu-109, that participate in substrate binding.

Fig. 8 shows the monomer structure of ClpB from Thermus thermophilus (8). The N-terminal domain is located at the top of the figure. Flexible diaphragms extending into the central channel in the oligomeric ClpB could not be resolved in the structure, but their positions are indicated by the flanking residues Glu-246 and Gln-651 in purple. The top diaphragm contains Tyr-251 that was mutated in the experiment shown in Fig. 7, and its position defines the entrance to the channel. The ClpB channel continues through the second diaphragm at Gln651 toward its exit at the C-terminal surface of the protein (bottom of Fig. 8). As shown in this monomer structure, the Asp-103 and Glu-109 pair is located at the surface of the N-terminal domain and faces the channel entry. In the oligomeric ClpB, the ring of N-terminal domains could create a funnel-like surface involved in binding aggregated polypeptides and guiding an exposed terminus of the substrate toward the channel entrance where Tyr-251 resides.

In the crystal structure of ClpB from T. thermophilus, the orientation of the N-terminal domain varied among crystallized monomers (8), which demonstrates high mobility of the link between the N-terminal domain and the first AAA+ module. It is possible that different orientations of the N-terminal domain help the chaperone seek and bind the recognition sites on the surface of protein aggregates. Whether the rotations of the N-terminal domains in oligomeric ClpB occur in a concerted way and whether they are coupled to the ATP-hydrolysis cycle of the AAA+ modules remains to be investigated. It is also possible that the N-terminal domain contains multiple substrate-recognition sites, as has been postulated for ClpA (31) and rotates to expose a site with the highest affinity for a particular substrate.

Fig. 9 shows the structure of the N-terminal domain of ClpB from E. coli (18). The two conserved acidic residues investigated in this work (Asp-103 and Glu-109) reside at the surface of the N-terminal domain in close proximity of Thr-7, which has been also identified as an essential
residue for the ClpB chaperone activity (14) and Phe-105, which is conserved among ClpB and yeast Hsp104. A group of conserved hydrophobic amino acids (green) forms a groove adjacent to the site formed by Thr-7, Arg-103, Phe-105, and Glu-109. It is possible that the region of the N-terminal shown in Fig. 9 represents a substrate-binding site capable of using a combination of electrostatic and hydrophobic forces to attract protein aggregates. The role of Asp-103 and Glu-109 in substrate binding is consistent with the propensity of ClpB to bind positively charged peptides (13).

Interestingly, whereas the folding topology of the N-terminal domain is similar in ClpB and ClpA (31), the latter protein contains an arginine in place of Asp-103 and an alanine in place of Glu-109, which may account for differences in substrate recognition between ClpA and ClpB.

This work also helps explain a discrepancy between previously published results on the chaperone activity of the full-length ClpB and ClpBΔN. Goloubinoff and co-workers showed that small protein aggregates were reactivated by the DnaK/DnaJ/GrpE system alone, but large aggregates also required ClpB for efficient reactivation (27). In previous studies on ClpB, whenever protein aggregates could be reactivated by the DnaK/DnaJ/GrpE system alone, the difference in activity between ClpB and ClpBΔN was small (21). Conversely, when a strong inhibition of ClpB activity upon the deletion of the N-terminal domain was observed, the aggregates were not reactivated by DnaK/DnaJ/GrpE alone (17). ClpBΔN does bind to the aggregates, but its binding affinity drops significantly as the aggregate size increases (see Fig. 6). Unlike in this work, no analysis of aggregate sizes was performed in previously published studies. In our opinion, which is supported by Fig. 2, small aggregates could have predominated in the former (21) and large aggregates in the latter (17) experiments. Why would an aggregate size matter for their recognition by a chaperone? Larger aggregates have a lower surface-to-volume ratio than smaller ones and may, therefore, expose a lower concentration of chaperone-binding sites (per mg of protein). Further studies on the mechan-anism of substrate recognition by ClpB will help clarify this result.

Recently, it has been shown that small heat-shock proteins are the third essential component of the aggregate-reactivation machinery besides DnaK/DnaJ/GrpE and ClpB (32, 33). Protein aggregates bound to small Hsps interacted with the full-length ClpB trap as well as with ClpBΔN trap (22). This result suggests that small Hsps may provide the “missing” aggregate-binding affinity to ClpBΔN in the absence of the N-terminal domain. The concerted action of DnaK/DnaJ/GrpE and small Hsps, which prevents the formation of large aggregates before their reactivation becomes necessary, may explain why either the full-length ClpB or ClpBΔN supports the survival of bacteria under heat shock (20, 34). However, a loss of ClpBΔN activity, but not that of the full-length ClpB is observed in vivo in the background of a defective DnaK.4

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Micheal E. Barnett, Maria Nagy, Sabina Kedzierska and Michal Zolkiewski

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