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Transglutaminase catalyzes differential crosslinking of small heat shock proteins and amyloid- β

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Abstract Crosslinking of proteins by tissue transglutaminase (tTG) is enhanced in amyloid (A β) deposits characteristic of Alzheimer's disease and sporadic inclusion body myositis. Small heat shock proteins (sHsps) also occur in amyloid deposits. We here report the substrate characteristics for tTG of six sHsps. Hsp27, Hsp20 and HspB8 are both lysine- and glutamine-donors, α B-crystallin only is a lysine-donor, HspB2 a glutamine-donor, and HspB3 no substrate at all. Close interaction of proteins stimulates crosslinking efficiency as crosslinking between different sHsps only takes place within the same heteromeric complex. We also observed that α B-crystallin, Hsp27 and Hsp20 associate with A β in vitro, and can be readily crosslinked by tTG.

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Keywords: Transglutaminase; Small heat shock protein; Crosslinking; Amyloid- β ; Protein aggregate

1. Introduction

Transglutaminases (TGs) are calcium-dependent enzymes catalyzing the formation of isopeptide bonds between the ϵ -amino group of polypeptide-bound lysines and the γ -carboxamide group of polypeptide-bound glutamines [1–3]. The ubiquitously expressed tissue TG (TGase2 or tTG) has been studied most extensively. Its substrate preference is not yet fully understood, but a common notion is that tTG is much less selective towards lysine residues than to glutamines. Both the adjacent residues in the primary structure and the exposure in the conformation of a protein determine whether glutamine and lysine residues can be reactive [2–5]. Tissue TG is involved in a wide array of biological activities, including differentiation and apoptosis as well as extracellular matrix remodeling [3,6,7]. As a result of increased tTG activity, large amounts of isopeptide crosslinks have been found in pathological deposits

of aggregated proteins such as amyloid (A β) [1,8,9]. Accumulation of A β is one of the major pathologic hallmarks of neuromuscular disorders like Alzheimer's disease (AD) and sporadic inclusion body myositis (sIBM), and A β is a well known substrate of tTG [10]. Whether tTG-catalyzed crosslinking is a cause or a consequence of the formation of aggregates is not clear.

Two members of the small heat shock protein (sHsp) family, α B-crystallin and Hsp27, have been reported as lysine-donor substrates for tTG [11,12]. In human, eight additional sHsps have been identified [13,14]. sHsps generally form large heteromeric complexes which readily exchange subunits [15,16]. Most sHsps are abundantly expressed in contractile tissues and to a lesser extent in the nervous system, kidney and skin [13,17]. As components of the cellular chaperone machinery, sHsps may bind to unfolding or aggregating proteins to keep them from further aggregation. α B-Crystallin has been found to be associated with the A β -containing senile plaques in AD brains [18] and with intracellular A β -aggregates in sIBM muscle fibers [19]. As a result of pathological overlap, the eye lenses of AD patients also show co-localization of α B-crystallin with A β -aggregates [20]. In addition, α B-crystallin can associate with A β in vitro [21], and increase its neurotoxic effect [22].

Since tTG, sHsps and A β co-localize at senile plaques in AD brains and inclusions in sIBM, we further explored the substrate characteristics of sHsps for tTG and the tTG-mediated crosslinking of sHsps and A β . We here report that in addition to α B-crystallin and Hsp27, also Hsp20 (or HspB6), HspB8 (also known as Hsp22 or H11) and HspB2, but not HspB3, are substrates for tTG. We found evidence that tTG-mediated crosslinking between different sHsps is more efficient when they can interact in the same mixed complex. In line with this result, we show that sHsps when interacting with A β can also efficiently be crosslinked to this peptide by tTG.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

The following constructs were used: pET3b-human- α B-crystallin, pET3a-human-Hsp27, pET8c-rat-Hsp20, pET16b-human-HspB8 and pET3a-rat-HspB2/B3 (both B2 and B3 cloned into the same vector). Protein expression was induced in the BL21 rosetta strain by addition of 350 μ M IPTG and subsequent incubation for three more hours at 37 °C. Cells were lysed by sonication in TEG buffer (25 mM Tris, pH

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Abbreviations: A β , amyloid- β ; AD, Alzheimer's disease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; sHsp, small heat shock protein; sIBM, sporadic inclusion body myositis; TBS, Tris-buffered saline; tTG, tissue transglutaminase

8.0, 2 mM EDTA, 50 mM glucose) and then centrifuged at $16000\times g$ for 45 min at 4 °C. Supernatants containing Hsp27, α B-crystallin and HspB2/B3 were subjected to 0.12% polyethylene-imine precipitation, and HspB8 to ammonium sulfate precipitation. All sHsps, except HspB8, were first fractionated on a DEAE–Sephacrose column, using a NaCl gradient from 0 to 1000 mM in 2 mM EDTA, 25 mM Tris–HCl/bis-Tris–HCl, pH 8.0–6.5 (depending on the pI of the sHsp), and subsequently purified on a Source 15Q HR 16/10 column. HspB8 was directly applied on Source 15Q. In each case, the last purification step was done on a Superose 6 gel permeation column using 25 mM bis-Tris, 2 mM EDTA, 150 mM NaCl, pH 7.0.

2.2. tTG-mediated crosslinking of small heat shock proteins

Two N-terminally biotinylated hexapeptides, biotin-GQDPVR (Q-donor) and biotin-GNDPVK (K-donor) (provided by Dr. J.W. Drijfhout, Leiden), were used as probes to detect tTG-catalyzed crosslinking. sHsps and tTG were first preincubated for 15 min at 37 °C in crosslinking reaction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 15 mM DTT and 20% glycerol). The crosslinking reaction was performed in a mixture with a final volume of 50 μ L containing 20 μ g sHsp, 1 μ g probe or 5 μ g $\text{A}\beta_{(1-40)}$ (Anaspec) and either no or 5 μ g (2.5×10^{-4} U/ μ L) guinea pig liver tTG (Sigma), and incubated for 2 h at 37 °C after adding CaCl_2 to a final concentration of 5 mM. Samples were analyzed by SDS–PAGE followed by electro-blotting and stained with Extravidin–peroxidase conjugate (Sigma) or the appropriate antibodies (see below).

2.3. sHsp– $\text{A}\beta$ association assay

$\text{A}\beta_{(1-40)}$ (5 μ g) was incubated either in the absence or presence of each sHsp (20 μ g) in 50 μ L crosslinking buffer without DTT for six days at 37 °C. After SDS–PAGE, samples were analyzed by Western blotting.

2.4. Brain sample preparation

Brain samples (obtained from the Department of Neurology, St. Radboud Hospital, Nijmegen) from autopsy material were homogenized and sonicated on ice in buffer (2% SDS, 150 mM NaCl, 100 mM Tris–HCl, 1 mM EDTA, 1 mM PMSF, Boehringer protease cocktail, pH 7.4). Samples were cleared by centrifugation and the collected

supernatant was analyzed by Western blotting. Protein concentration was determined by the BCA kit (Pierce) according to the manufacturer's manual.

2.5. SDS–PAGE and Western blotting of recombinant proteins and brain materials

For the detection of proteins the following antibodies were used: anti- $\text{A}\beta$ (Anaspec), anti- α B-crystallin (Riken Cell Bank), anti-Hsp27 (Stressgen), rabbit anti-Hsp20 and anti-actin (Sigma). Samples containing 20 μ g of brain proteins or 0.2–2 μ g of sHsps present in crosslinking reaction mixture were subjected to SDS–PAGE and transferred to nitrocellulose membranes (Hybond). Membranes were then blocked with 5% non-fat dried milk in TBST (15 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and probed with the appropriate primary and secondary antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL). Western blotting detection kit (Pierce) or alkaline phosphatase (Promega) reaction.

3. Results

3.1. In vitro crosslinking of sHsps as substrates for transglutaminase

We examined the six prominent members of the mammalian sHsp family which are expressed throughout the body. To determine the presence of glutamine and lysine donor sites for tTG in these sHsps, we assayed their capacity to be crosslinked to the peptides biotin-GNDPVK and biotin-GQDPVR, respectively. These peptide sequences are derived from the skin protein SKALP, which is known to be an excellent tTG substrate [23].

In agreement with earlier findings [11,24], Fig. 1 shows that α B-crystallin is a potent K-donor and has no Q sites available for tTG. Also Hsp27 turns out to be an efficient K-donor (Fig. 1), as expected on basis of earlier findings with Hsp25, the

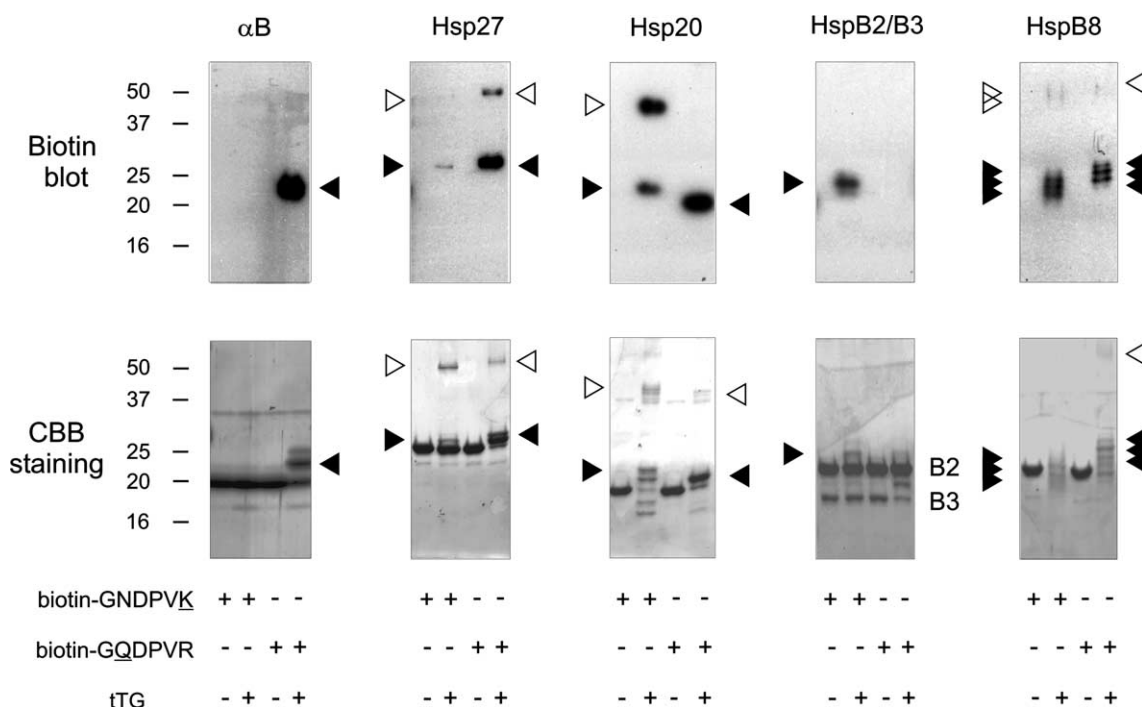


Fig. 1. Small heat shock proteins as substrates for tTG. Twenty μ g of each recombinant sHsp was incubated with the biotin-GNDPVK and biotin-GQDPVR probes at 2 μ M final concentration, in the absence or presence of 2.5×10^{-4} U tTG/ μ L. Samples were then analyzed by SDS–PAGE followed by biotin blotting (upper panels) or staining with CBB (lower panels). Arrowheads (\blacktriangleleft) and open arrowheads (\triangleleft) indicate the monomeric and dimeric forms of sHsps crosslinked to the probes, respectively.

mouse ortholog of human Hsp27 [12]. However, Hsp27 in addition shows some Q-donor reactivity, which explains that Hsp27 in contrast to α B-crystallin can crosslink into a homodimer (open triangles, Fig. 1). The Hsp27-dimer could still be labeled with both probes (Fig. 1, Hsp27, upper panel) indicating that the available but unused K and Q sites are still accessible for tTG.

Similar to Hsp27, labeling of recombinant rat Hsp20 resulted in incorporation of both the K- and Q-probes. Hsp20 seems to be a very good substrate for tTG, since almost all Hsp20 underwent tTG-catalyzed modifications (Fig. 1, Hsp20, lower panel, second and fourth lanes). A considerable proportion of Hsp20 formed homodimers, especially in the presence of the K-donor probe. Non-labeled bands migrating faster than the unmodified monomer of Hsp20 were also found upon tTG incubation (Fig. 1, Hsp20, lower panel). This might be due to the formation of intramolecular isopeptide bonds, resulting in a circular polypeptide.

HspB2 forms a stable complex with HspB3 [25] and for this reason they were expressed together in *Escherichia coli* and purified as a complex. No incorporation of the K- or Q-donor probe was detected in HspB3, but we obtained a good labeling of HspB2 with the K-donor probe (Fig. 1, HspB2/B3 panel). In agreement with the absence of K-donor sites in HspB2, no dimers were observed.

Finally, HspB8 underwent extensive modification upon tTG treatment, leaving hardly any unmodified protein (Fig. 1, HspB8, lower panel). Labeling with both the Q- and K-probes yielded several bands (upper panel), indicating the presence of multiple accessible Q- and K-donor sites. Bands at the expected position of the dimers were detected only faintly on both the biotin blot and the corresponding CBB-stained gel.

3.2. Crosslinking of interacting subunits of sHsps

Crosslinking catalyzed by tTG may occur between subunits within the same complex as well as between subunits present in different complexes, depending on localization and accessibility of K- and Q-donor residues. To distinguish between these possibilities, we made use of the observation that HspB2 and HspB3 are able to form mixed complexes with Hsp20, but not with Hsp27 or α B-crystallin [25]. How HspB8 interacts with other sHsps is as yet not known, and HspB8 is therefore not

included in these crosslinking studies. Hsp27, Hsp20 and α B-crystallin were first preincubated at 37 °C, either alone or with other sHsps, to allow exchange of subunits and the formation of mixed complexes. Tissue TG was subsequently activated by the addition of calcium (Fig. 2). As already observed in Fig. 1, Hsp27 and Hsp20 alone, but not α B-crystallin, could form homodimers. Crosslinking of premixed Hsp27 and Hsp20 yielded beside the homodimers also heterodimers (a in panels A and B, Fig. 2), indicating that Hsp27 and Hsp20 can crosslink to each other. Interestingly, α B-crystallin premixed with Hsp27 or Hsp20 could also form heterodimers (b in panels A and C, and d in panels B and C, respectively, Fig. 2), showing that the available K-donor site of α B-crystallin is sufficient for crosslinking with Hsp27 and Hsp20. However, HspB2 was only found to be crosslinked to Hsp20 (c in panel B, Fig. 2), whereas no crosslinking was observed with Hsp27 or α B-crystallin. It thus appears that sHsp subunits can more readily be crosslinked when they are interacting in the same heteromeric complex than when they are present in different homomeric complexes.

3.3. Crosslinking of sHsps to A β

Several studies have demonstrated in various experimental systems the interaction between sHsps and A β [21,22,26,27]. Such interactions are physiologically relevant only if the particular sHsps are present in AD brains. We therefore identified the sHsps that are present in autopsy material from temporal cortices of three AD patients and three control individuals by Western blotting. We found variable levels of Hsp27, α B-crystallin and Hsp20 in both control and AD brain samples, and no indication for increased expression in the latter (Fig. 3). No expression of HspB2, HspB3 and HspB8 could be detected by immunoblotting (data not shown). In line with previous investigations [8], AD brains showed slightly higher expression of tTG as compared to controls.

To analyze the possible association between A β and the three sHsps that are present in brain, A β was incubated for 6 days at 37 °C with the sHsps and subsequently separated on SDS-PAGE and identified by immunoblotting (Fig. 4). In the absence of sHsps, A β was mainly present as SDS-resistant aggregates in the form of a smear and practically undetectable in its monomeric form. Co-incubation with α B-crystallin,

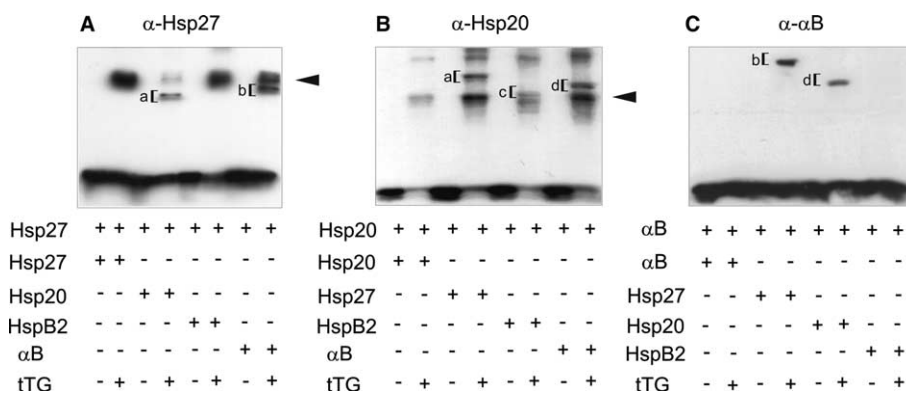


Fig. 2. Transglutaminase catalyzed crosslinking of interacting subunits of sHsps. Forty μ g of sHsps (20 μ g of each) were mixed with 2.5×10^{-4} U tTG / μ L. Subunit exchange was facilitated by preincubation at 37 °C, after which tTG was activated by the addition of 5 mM CaCl₂. After crosslinking, the samples were separated by SDS-PAGE and immunoblotted with antisera against Hsp27 (panel A), Hsp20 (panel B) α B-crystallin (panel C). Arrowheads indicate the homodimers and brackets with letters the heterodimers: (a) Hsp20–Hsp27; (b) Hsp27– α B; (c) HspB2–Hsp20; (d) Hsp20– α B. Since HspB3 is not a substrate for tTG, no crosslinking of HspB3 was observed with other sHsps.

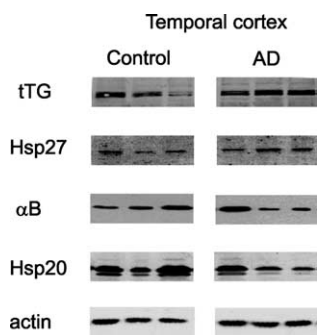


Fig. 3. Transglutaminase and sHsp expression in the temporal cortex of control and AD patients. Extracts containing 20 μ g of protein from cortical brain tissue of three healthy controls and three AD patients were separated by SDS–PAGE and subsequently analyzed by Western blotting with antibodies directed to tTG, Hsp27, α B-crystallin and Hsp20, as indicated. Actin was used as an internal control.

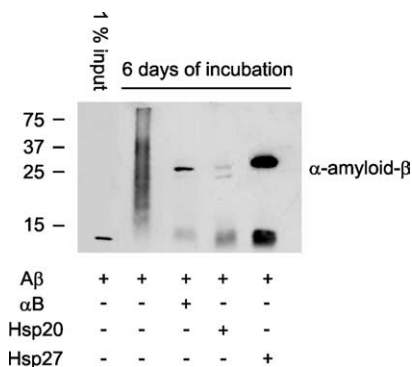


Fig. 4. sHsps induce the formation of specific oligomers of $A\beta_{1-40}$. Five μ g of $A\beta$ was incubated either in the absence or presence of sHsp in crosslinking buffer without DTT, for six days at 37 °C. Samples then were subjected to immunoblotting and stained with a polyclonal antibody against $A\beta$. Lane 1 represents 1% of the input, lanes 2–5 represent the samples after incubation of $A\beta_{1-40}$ for 6 days without sHsp or with α B-crystallin, Hsp20 and Hsp27, respectively. The stacking gel has been removed prior to blotting; any fractions not penetrating the separating gel may thus have been missed.

Hsp20 or Hsp27 drastically changed the polymerization properties of $A\beta$. In the presence of these sHsps, part of $A\beta$ remained in the monomeric form and part of it formed multimers with masses between 22 and 28 kDa. These multimer bands did not counterstain with anti-sHsps antibodies (data not shown). Thus, these sHsps appear to be able to inhibit the aggregation of $A\beta$ by promoting the formation of smaller-sized SDS-resistant intermediates.

Next, we determined whether tTG could crosslink $A\beta$ to Hsp27, α B-crystallin and Hsp20. The sHsps were incubated for 2 h at 37 °C in the presence of calcium either with or without tTG and $A\beta$. Upon incubation with tTG, we found all three sHsps strongly decorated with $A\beta$ -monomers (Fig. 5, last lane in each panel). On the blots probed with anti- $A\beta$ -antibody a slower migrating band was detected above the main cross-linked products (Fig. 5, upper panels, top black arrowhead), indicating that more than one monomer of $A\beta$ could be crosslinked to these three sHsps. In addition, crosslinking of $A\beta$ to Hsp20 and Hsp27, but not α B-crystallin, also resulted

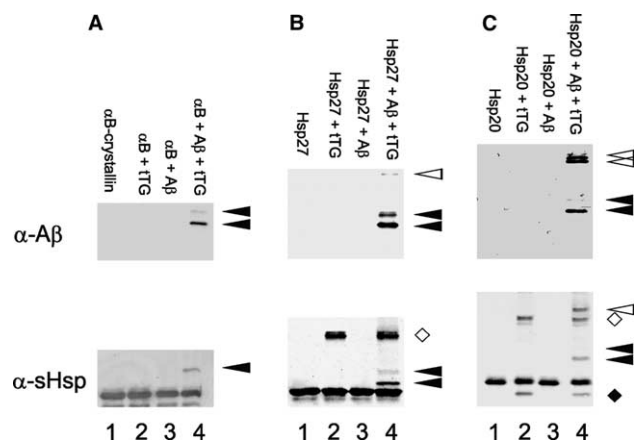


Fig. 5. sHsps can readily crosslink to $A\beta_{1-40}$. sHsps (0.1 μ g/ μ L) were incubated for 2 h at 37 °C alone (lanes 1) in the presence of tTG (50 ng/ μ L) (lanes 2), in the presence of $A\beta_{1-40}$ (0.1 μ g/ μ L) (lanes 3), or together with tTG (50 ng/ μ L) and $A\beta_{1-40}$ (0.1 μ g/ μ L) (lanes 4). Samples were separated by SDS–PAGE and subjected to Western blotting with anti- $A\beta_{1-40}$ (top panels) and anti-sHsp antibodies (bottom panels). Panels A, B and C show the crosslinking of $A\beta_{1-40}$ to α B-crystallin, Hsp27 and Hsp20, respectively. Crosslinking products of $A\beta_{1-40}$ with sHsp monomers are indicated by black arrowheads (\blacktriangle), with sHsp dimers by open arrowheads (\triangleleft), intermolecularly crosslinked Hsp27 and Hsp20 is marked by open diamonds (\diamond), and intramolecularly crosslinked Hsp20 by a filled diamond (\blacklozenge).

in $A\beta$ -decorated sHsp dimers (Fig. 5, upper panels, open arrowheads).

4. Discussion

Earlier studies have shown that both α B-crystallin and Hsp25 expose a lysine residue accessible for tTG [11,12,24]. Using biotinylated hexapeptide probes for K- or Q-donor sites, we screened for additional tTG substrates in the sHsp family (Fig. 1). These labeling experiments revealed that crosslinkable lysine residues were not restricted to α B-crystallin and Hsp27, but are also present in Hsp20 and HspB8. For both α B-crystallin and Hsp27, it has been shown that only the C-terminal lysine is available for tTG [12,24]. The C-terminal flexible tail of Hsp20 also exposes a terminal lysine and it is thus likely that this lysine is a glutamine acceptor. HspB8 clearly serves as a multiple lysine-donor for tTG although it lacks a C-terminal lysine. Therefore, the lysine substrates of this protein must be localized elsewhere.

There are only few glutamine substrates known in intracellular proteins. Surprisingly, we found that Hsp27, Hsp20, HspB2 and HspB8, but not α B-crystallin, all exposed glutamines to tTG. Crosslinked Hsp27 has been identified very recently in neurofibrillary tangles which strongly corroborates our data about the glutamine site exposure in Hsp27 [28]. The absence of a glutamine-donor in α B-crystallin is in agreement with earlier studies [11,24], but not with the reported formation of homodimers by bovine lens α B-crystallin in the presence of tTG [29]. This discrepancy suggests that differences in the isolation or incubation procedures may affect the accessibility of substrate sites for tTG. The lower panel of Fig. 1 shows that Hsp20 and HspB8 are the most vulnerable to tTG modification, indicating that both sHsps expose multiple

substrate sites for intra- and intermolecular crosslinking. In the case of HspB8, this nicely correlates with the recent observation that HspB8 displays a randomly coiled and labile structure [30].

Two groups of heteromeric sHsp complexes have been distinguished in muscle extracts, one comprising Hsp27, α B-crystallin and Hsp20, and the other HspB2 and HspB3 [25]. Apart from Hsp20, members of one group do not form mixed complexes with those from the other group of sHsps. However, in yeast two-hybrid analyses Hsp20 seems to behave differently and can bind to HspB2 [25]. Thus, Hsp20 might provide cross-talk between the two systems by its ability to associate not only with Hsp27 and α B-crystallin, but also with HspB2. Our crosslinking experiments support this observation. While α B-crystallin, Hsp27 and Hsp20 can readily crosslink with each other (a, b and d in Fig. 2), Hsp20 is the only protein that can also crosslink to HspB2 (c in Fig. 2). The tTG-catalyzed crosslinking of different sHsps thus seems to be facilitated by their interaction. This might also implicate that the aggregating proteins present in pathological inclusions become prone to be fixed by tTG-catalyzed protein-crosslinking.

Hsp27 and α B-crystallin have been described in senile plaques in AD and in amyloid inclusions in sIBM [18,19]. The major component of the plaques is A β , and this peptide has been shown to expose both Q and K sites for tTG [31]. The aggregation of A β could possibly trigger its association with sHsps [22]. Our co-incubation experiments of A β with Hsp27, Hsp20 and α B-crystallin indeed suggest that sHsps can associate with A β and more importantly, they may interfere with the polymerization process of A β by fixing it in a defined oligomeric form (Fig. 4). This observation correlates well with the recent finding that α B-crystallin reduces the amount of physiologically stable amyloid deposits in favor of easily degradable aggregates [32]. In the presence of tTG, we find that A β can readily crosslink to sHsps in vitro. However, in the AD material we examined no sHsp was seen crosslinked to A β (Fig. 3), but this might be due to insolubility of such products. Based on our observation, it is not unlikely that the crosslinking efficiency between A β and sHsps is stimulated by their association. This might be an important aspect of the amyloid disease process, since the amount of isopeptide crosslinks in AD brain and sIBM muscle is strongly increased, up to 30- to 50-fold [8,9].

In conclusion, the human sHsps that we investigated are excellent in vitro substrates of tTG. By binding to A β , sHsps seem to have the potential to arrest its polymerization process. Moreover, larger complexes, such as those formed when sHsps associate with unfolding and aggregating proteins may become stabilized by their crosslinking. This phenomenon could contribute to the formation of inclusion bodies in patients with Alzheimer's disease and sporadic inclusion body myositis.

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