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Expressed protein ligation for a large dimeric protein

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Expressed protein ligation (EPL) is a protein engineering tool for post-translational ligation of protein or peptide fragments. This technique allows modification of specific parts of proteins, opening possibilities for incorporating probes for biophysical applications such as nuclear magnetic resonance (NMR) or fluorescence spectroscopy. The application for oligomeric proteins, however, is restricted by the need to obtain a large excess of active dimer over reactants and intermediates. Here, we explored the suitability of the EPL reaction for large dimeric proteins using the molecular chaperone Hsp90 as a model. We systematically varied the reaction conditions and the preparation protocols for the reactants. Modulation of the ligation site by shortening the flexible segment at the N-terminus of the C-terminal reactant increased the yield sufficiently to isolate the product by chromatography. Under those conditions, 41% of the used C-terminal fragment could be successfully ligated. We discuss possible up-scaling for segmental isotope labelling for NMR applications.

Keywords: intein/molecular chaperones/protein engineering/protein folding/protein–protein interactions

Introduction

Expressed protein ligation (EPL) is the post-translational ligation of recombinant polypeptides (Muir, 2003). EPL makes use of the self-splicing property of proteins called inteins to achieve the post-translational chemical ligation of two or more segments of a protein under native conditions. Inteins catalyse the precise removal of an internal segment within a protein and ligate the two flanking regions, known as exteins (Xu and Perler, 1996; Paulus, 2000, 2001; Muir, 2003). Therefore, inteins can be artificially introduced into protein sequences for protein engineering purposes (Severinov and Muir, 1998; Blaschke *et al*., 2000).

One of the most important applications of EPL is the segmental isotope labelling of large molecules for nuclear magnetic resonance (NMR) spectroscopy (Heinamaki *et al*., 2009; Skrisovska *et al*., 2010). Segmental isotope labelling is the selective incorporation of NMR detectable isotopes in just a part of the protein. Protein NMR spectroscopy was typically restricted to proteins up to 30 kDa since transverse relaxation causes signal loss for large systems (Pervushin, 2000; Wüthrich *et al*., 2000). Recent advances in NMR spectroscopy increased the conventional NMR size limit. Transverse Relaxation Optimized Spectroscopy (TROSY) experiments improved sensitivity of NMR experiments to allow analysis of protein complexes up to 900 kDa (Pervushin *et al*., 1997; Otomo *et al*., 1999; Riek *et al*., 1999; Wüthrich *et al*., 2000). However, those improvements do not deal with the spectral complexity resulting from the large number of signals that may overlap. The overlap of signals can be reduced by segmental isotope labelling of the specific domains of the protein (Evans *et al*., 1999; Otomo *et al*., 1999; Xu *et al*., 1999; Camarero *et al*., 2001).

The application of EPL for NMR purposes is particularly attractive for multimeric proteins since even most dimers exceed the traditional NMR size limits. Ligation of dimers demands two key requirements for the EPL chemistry: (i) The EPL reaction must be rather efficient to prevent an excess of heterodimers of ligated product with unligated reactant. (ii) The reaction mixture needs effective separation of the product from both incomplete intermediates and reactants. These two conditions are particularly difficult for EPL of oligomeric proteins. In case the efficiency is low, this would lead to an excess of heterooligomers next to little fully ligated product. This is especially unwanted for expensive, isotope-labelled NMR samples and it also complicates purification of the desired product.

We set out to explore the EPL reaction for a large dimer, using the molecular chaperone Hsp90 as a model. We optimised reactant concentration, pH, proteolytic cleavage and domain boundaries, and monitored the effect on the EPL yield of Hsp90. Crucial for high yields was the removal of a flexible linker at the ligation site. We separated ligated Hsp90 dimer from heterodimers and non-reacted species by heparin affinity chromatography. Our study shows that EPL is suitable for generating large dimeric proteins, which makes them accessible for segmental isotope labelling and future NMR studies.

Results

Intein-mediated protein ligation succeeded for Hsp90

The EPL reaction requires an intein at the C-terminus of the N-terminal fragment and a free cysteine at the N-terminus of the C-terminal fragment to be ligated (Fig. 1). Hsp90 consists of three domains, an N-terminal ATPase, a middle domain without enzymatic function and a C-terminal domain involved in the formation of Hsp90 homodimers (Pearl and Prodromou, 2006). The best candidate for cutting the protein into two halves is a long, non-conserved flexible charged linker between the N-terminal and middle domains, which was shown to be dispensable in crystal structures of Hsp90 homologues (Ali *et al*., 2006; Dollins *et al*., 2007). There was, however, no native cysteine present within the flexible charged linker of Hsp90. We introduced, therefore, a cysteine

by site-directed mutagenesis. The efficiency of the EPL is known to improve when the active cysteine is followed by a glycine to increase flexibility (Xu and Evans, 2001). For that reason, we chose a position N-terminal of a native glycine residue in the flexible linker to minimise the extent of protein modification, resulting in the C-terminal fragment Hsp90-K233C-D723 (Fig. 2A).

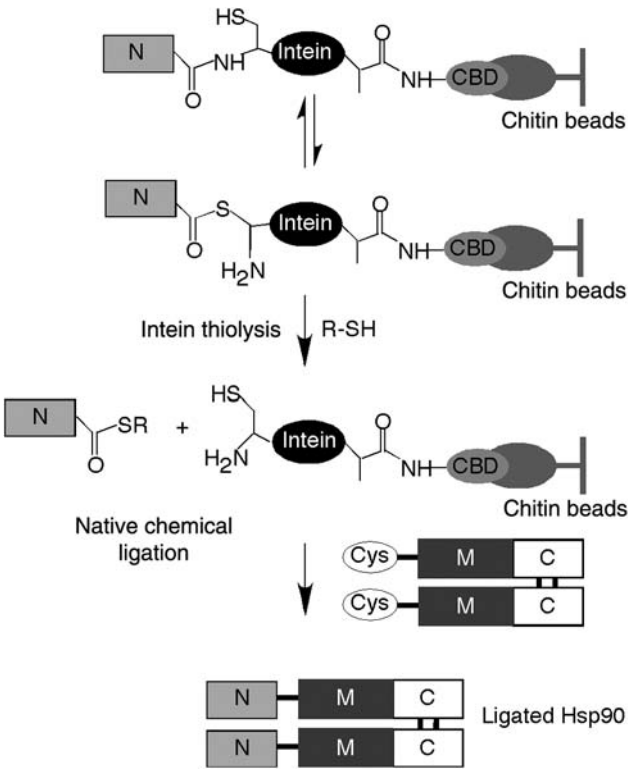

Fig. 1. Schematic description of the EPL reaction of Hsp90. Hsp90-N (Hsp90-P1-E232) is expressed as fusion protein of intein and chitin binding domain (CBD). N-terminal cleavage of intein induced by a thiol compound generates an active Hsp90-N with a C-terminal thioester. The complementary sequence Hsp90-MC (Hsp90-K233C-D723) bears an N-terminal cysteine introduced with site-directed mutagenesis. Protein–protein ligation occurs via a chemoselective reaction and a subsequent spontaneous S–N acyl rearrangement to form a peptide bond between the reacting species.

We expressed the complementary N-terminal fragment Hsp90-P1-E232 as C-terminal Mxe GryA intein fusion protein (Fig. 2A), followed by a chitin binding domain (CBD) for easy purification, following the work of Xu and co-workers (Chong *et al.*, 1997). This Mxe GyrA intein construct encodes for a mutated intein that can undergo protein splicing. The fusion of both fragments by EPL reaction would result in a full-length Hsp90 protein free of any purification tag, with a K233C mutation in the non-conserved flexible charged linker (Fig. 2A). The mutation of the lysine residue to cysteine is not expected to have significant impact on Hsp90 function as the replacement of the residues in the flexible charged linker to serine–glycine stretch was shown not to change the activity of Hsp90 (Hainzl *et al.*, 2009).

To be able to produce a C-terminal fragment with an N-terminal cysteine, we introduced a Factor-Xa proteolytic cleavage site between the hexa-His purification tag and the cysteine residue. Application of the C-terminal fragment onto nickel (Ni)-NTA-agarose beads, followed by its elution from the Ni-NTA-agarose beads by Factor-Xa cleavage, yielded in the C-terminal fragment with the N-terminal cysteine. We further purified the fragment on an anion exchange column and concentrated it to 50 μM.

The N-terminal fragment was pre-purified via anion exchange chromatography, followed by loading the eluate onto chitin beads, which allowed the further purification of the N-terminal fragment. We first saturated the chitin beads with the N-terminal fragment, which results in 46 μM approximate concentration of N-terminal fragment on the beads. After washing away all contaminants, we performed the reaction with N-terminal fragment still bound to the column, as this step is known to increase the efficiency of the reaction (Welker and Scheraga, 1999; Sydor *et al.*, 2002; Anderson *et al.*, 2005; Zhao *et al.*, 2008). We then loaded the C-terminal fragment onto the chitin column in the presence of 50 mM 2-mercaptoethanesulphonic acid (MESNA) to initiate EPL. We allowed the reaction to take place under continuous rotation for up to 2 weeks at 4°C.

EPL with Hsp90-P1-E232 and Hsp90-K233C-D723 did yield full-length Hsp90 protein (Fig. 3A), but the efficiency of the reaction was not high enough to produce protein at

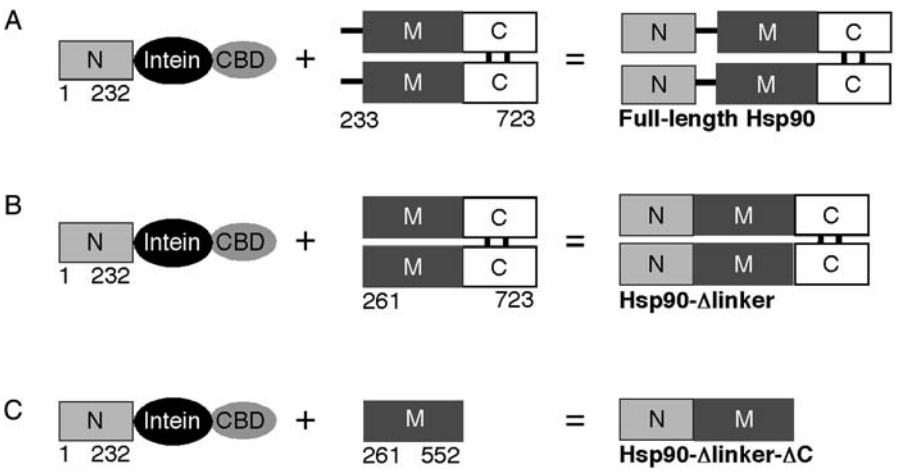

Fig. 2. Schematic representation of EPL products created in this study. (**A**) Synthesis of full-length human Hsp90*β*, residues P1-E723, with a K233C point mutation. (**B**) Hsp90-Δlinker, construct as in (A), but with a S260C point mutation and lacking residues K233-K259. (**C**) Hsp90-Δlinker-ΔC, construct comprising residues P1-M552 with a S260C point mutation and lacking the segment comprising residues K233–K259.

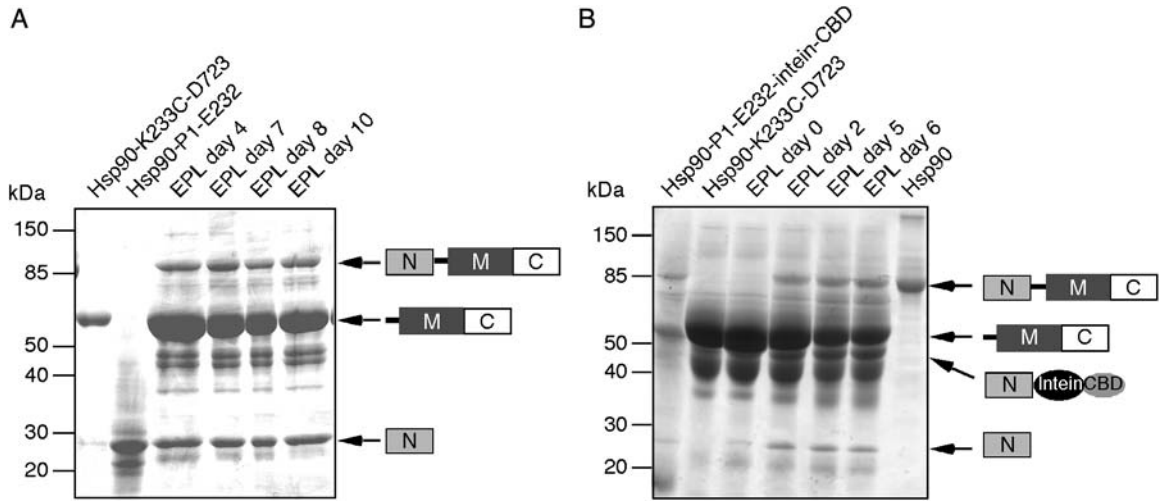

Fig. 3. The EPL reaction forms full-length Hsp90. (**A**) The SDS–PAGE gel of EPL reaction between Hsp90-P1-E232 and Hsp90-K233C-D723 fragments displaying samples from days 4, 7 and 8 and 10th day of the reaction. The reaction was performed with 55 nmol Hsp90-P1-E232 bound to chitin beads and 10 nmol of Hsp90-K233C-D723 (50 μM final concentration) at pH 8.0, 10 μl of EPL reaction mixture were loaded in each lane on the gel. (**B**) The EPL reaction with increased concentration of Hsp90-K233C-D723. The SDS–PAGE gel of EPL reaction performed with 32 nmol of Hsp90-K233C-D723 (160 μM final concentration), conditions as described for (A). Five microlitres of EPL reaction mixture were loaded in each lane of the gel.

NMR scale. An effective reaction is essential to ensure formation of sufficient amounts of homogeneous full-length Hsp90 dimer. Several factors may affect efficiency, such as concentration of reactants, eventual steric hindrance due to dimerisation of Hsp90's C-terminal fragments, contaminants, pH or temperature.

EPL efficiency did not increase with increasing the concentration of the C-terminal fragment

The EPL is a bimolecular reaction. The increase in the protein concentration therefore should increase reaction efficiency. The maximal concentration of the N-terminal fragment is limited by the capacity of the chitin beads, which were saturated with N-terminal fragment. The concentration of the C-terminal fragment can be varied, though. We increased the concentration of the C-terminal fragment Hsp90-K233C-D723 from 50 to 160 μM, but this failed to significantly increase the reaction yield (Fig. 3B). We observed that the reaction reached a plateau after 2 days with no significant increase in the product afterwards.

The SDS–PAGE of Hsp90-K233C-D723 showed a few contaminating bands after purification. It was not possible to separate those contaminants from Hsp90 after rigorous washing on the Ni-NTA-agarose beads followed by anion exchange chromatography. Hsp90 molecule is prone to proteolysis at a predominant cleavage site in the C-terminal dimerisation domain and at the junction of the middle domain and the flexible charged linker (Tsutsumi *et al.*, 2009). During its expression in bacteria or Factor-Xa treatment, Hsp90 might have been cleaved non-specifically at those sites. The contaminants might be N-terminal fragments of Hsp90-MC that possess the hexa-His tag or the C-terminal domain that can still dimerise with Hsp90-MC. Only N-terminal fragments of Hsp90-MC could affect the efficiency of the reaction by reacting with N-terminal domain resulting in shorter Hsp90 products. However, it was not possible to separate those contaminants due to similar binding properties to the anion exchange column.

Changing pH did not increase EPL yield

EPL reactions are typically performed in the pH range of 7.0–8.0. Use of this pH window ensures that the ligation reaction is chemoselective (Muralidharan and Muir, 2006). To test the effect of the pH on reaction yields, we performed the ligation reaction with pH 7.0, 7.3, 8.0 and 8.5. The results indicate no significant difference in the efficiency of EPL in those conditions (Fig. 4A and B). However, at pH 8.5 the rate of cleavage of the thioester bond between the N-terminal Hsp90 fragment and intein was reduced, judged by the lower levels of N-terminal fragment (Fig. 4B). This indicates that the cleavage of the intein thioester is not rate limiting for EPL of Hsp90.

EPL yield was not limited by unspecific cleavage of the N-terminal cysteine

One of the reasons of the low EPL yield could be potential unspecific cleavage activity of Factor-Xa when preparing the C-terminal fragment with N-terminal cysteine residue for the reaction. Since Hsp90-K233C-D723 is cleaved at the N-terminus of a long flexible linker, Factor-Xa might also cleave downstream of its recognition site. Any cleavage that would remove the N-terminal cysteine would prevent the EPL reaction, and therefore it was mandatory to test this by preparing the C-terminal fragment in a protease-independent manner. We expressed the C-terminal fragment Hsp90-K233C-D723 as SUMO-fusion protein. Specific cleavage of SUMO-Hsp90 by SUMO-hydrolase Ulp1 generated Hsp90-K233C-D723. Despite the high specificity of Ulp1, the yield of the EPL did not improve (Fig. 5). Therefore, we conclude that the EPL yields had not been reduced by eventual unspecific cleavage activity of Factor-Xa.

The flexible charged linker decreased EPL efficiency

We tested whether the domain boundaries have an effect on the reaction efficiency. We designed a C-terminal fragment omitting the flexible linker in Hsp90 (Fig. 2B). We chose to introduce the cysteine in the place of S260 by site-directed

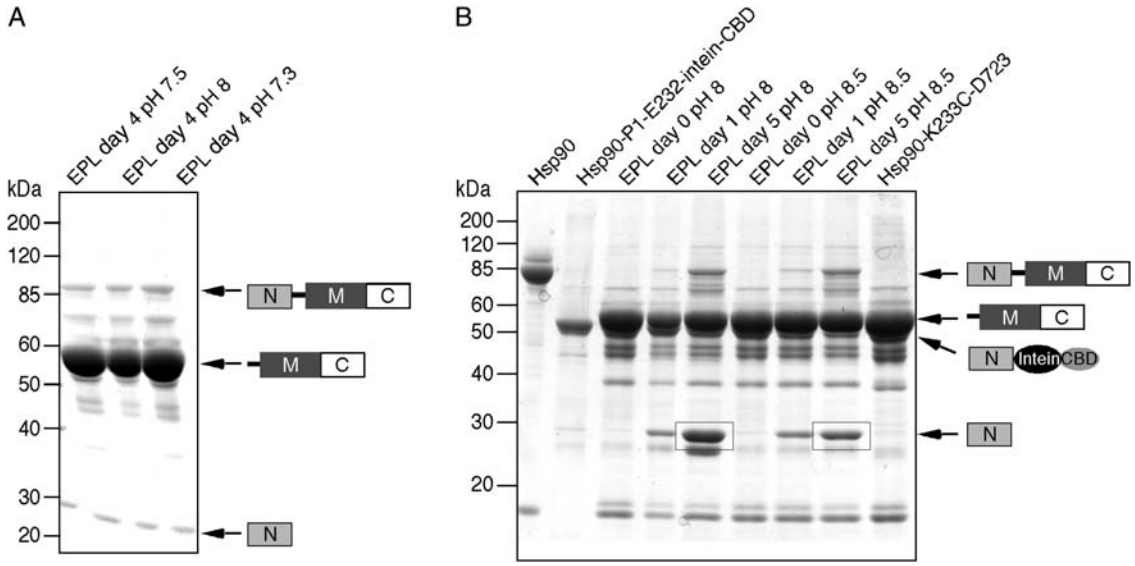

Fig. 4. The EPL reaction of Hsp90 performed at various pH. (**A**) The SDS–PAGE gel of EPL reaction carried out at pH values 7.3, 7.8 and 8.0, displaying the fourth day of the reaction; there was no further improvement in the reaction yield after 4 days. (**B**) SDS–PAGE gel displaying EPL reaction carried out at pH 8.0 and 8.5. The cleaved N-terminal fragment from intein is depicted with a small box.

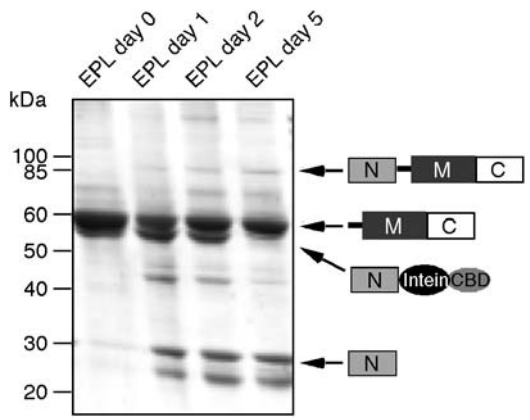

Fig. 5. Low EPL efficiency is not caused by the unspecific cleavage of N-terminal cysteine of Hsp90-K233C-D723. SDS–PAGE gel displaying samples of the EPL with Hsp90-K233C-D723 expressed as a SUMO fusion protein. Changing the protocol to obtain specific cleavage of Hsp90-K233C-D723 did not improve the amount of the ligated product.

mutagenesis. This region is not conserved in the Hsp90 sequence and S260 is positioned next to a glycine residue that is known to increase the efficiency of the reaction.

We now ligated intein-fused Hsp90-P1-E232 and Hsp90-S260C-D723 via EPL (Fig. 2B). It successfully led to production of Hsp90-Δlinker, in which the segment K233-D259 was removed. In fact, we obtained higher yields than previously with the longer fragment Hsp90-K233C-D723, which resulted in the production of full-length Hsp90 after the reaction (Fig. 6A).

To validate that the flexible linker is responsible for the difference in yield in the experiments described above, we repeated the EPL with both C-terminal fragments in parallel. This confirmed that under exactly identical circumstances the yield for Hsp90-S260C-D723 was considerably higher than for K233C-D723 (Fig. 6B). We concluded that Hsp90-K233C-D723 was less efficient in the EPL reaction because of its conformational flexibility at its N-terminus.

EPL was not hindered by dimerisation of the C-terminal fragment

A potential problem for the EPL reaction of Hsp90 might be steric hindrance due to dimerisation of the C-terminal fragment. We tested this by performing an experiment with truncated middle-domain Hsp90-S260C-M552 (Fig. 2C), which does not include the dimerisation domain of Hsp90. The intein-meditated ligation yielded a monomeric Hsp90-P1-M552-Δlinker fragment (Fig. 2C), in which the segment K233-D259 was lacking. We performed the ligation reaction under identical conditions as before, for the reaction of Fig. 6A. However, deleting the C-terminal dimerisation domain of the Hsp90 did not result in the increase of the EPL yield compared with the Hsp90-S260C-D723 fragment (Fig. 7). We concluded that the dimerisation at the C-terminus of Hsp90 does not affect the reaction yield by steric hindrance.

Separation of ligated Hsp90 from side products

The preparation of a homogenous product requires purification of ligated Hsp90 homodimers from several other fragments present in the EPL reaction mixture, including heterodimers of Hsp90-Δlinker with the C-terminal fragment, smaller Hsp90 fragments and eventual side products. The yield for Hsp90-Δlinker was sufficient to allow purification by chromatography, and to subsequently quantify the yield of the reaction. We subjected the mixture onto a heparin affinity column to separate the reaction mixture. This column successfully separated Hsp90 homodimers from reactants and side products (Fig. 8).

After purification we obtained 1.0 mg (0.012 μmol) of Hsp90-Δlinker fusion product from 2.0 mg (76.4 nmol) of Hsp90-P1-E233 and 1.6 mg (29 nmol) of Hsp90-S260C-D723. This corresponds to an isolated yield of 41%; with respect to Hsp90-S260C-D723. For Hsp90-P1-E233, which was used in excess, we estimated that 15% were incorporated in the final product. Our study shows that segmentally isotope-labelled Hsp90-Δlinker can be produced and purified

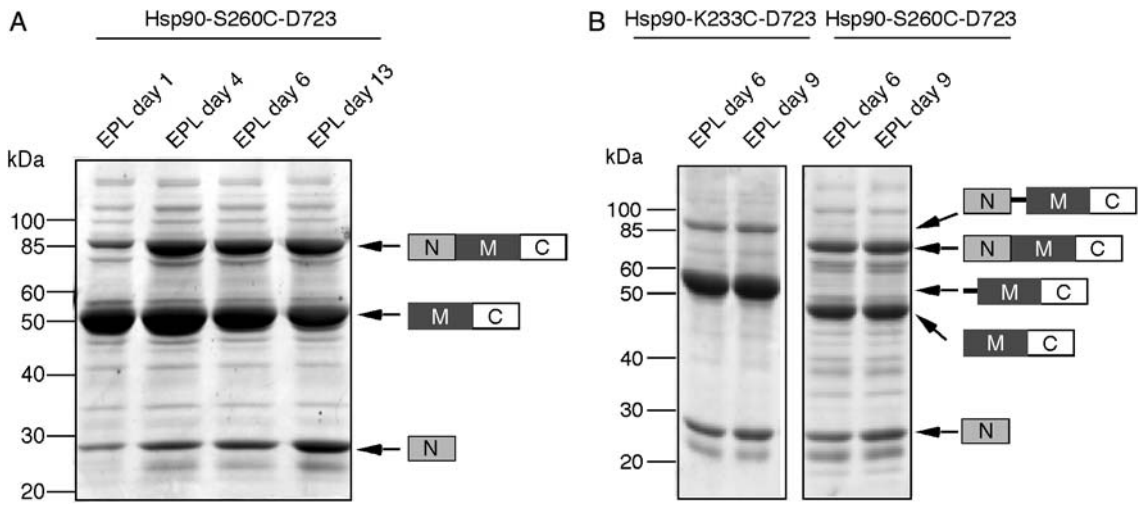

Fig. 6. The EPL efficiency increases significantly when the charged flexible linker is omitted. (**A**) SDS–PAGE gel showing EPL with Hsp90-S260C-D723. The reaction leads to formation of Hsp90-Δlinker in which the part of the flexible charged linker is left out. (**B**) SDS–PAGE gel of EPL reactions performed with Hsp90-MC with different boundaries.

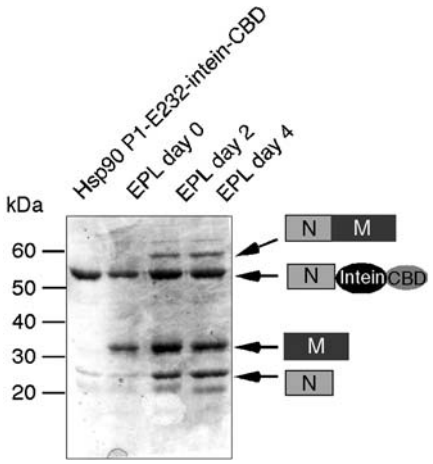

Fig. 7. The EPL reaction performed with C-terminal fragment lacking Hsp90 dimerisation domain. SDS–PAGE gel displaying samples of the EPL performed with Hsp90-S260C-M552. The EPL reaction yielded a monomeric Hsp90-P1-M552-Δlinker fragment, in which the segment K233-D259 was lacking.

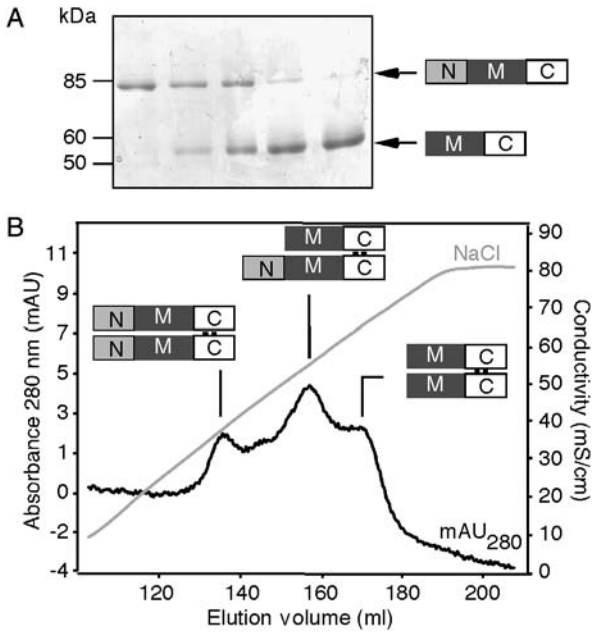

Fig. 8. Hsp90-Δlinker can be purified by heparin affinity purification after EPL. (**A**) The SDS–PAGE gel indicates the protein species present in the peaks. (**B**) Elution profile of heparin affinity purification of EPL reaction producing Hsp90-Δlinker. The Hsp90-Δlinker homodimer elutes first, followed by the second peak corresponding to the heterodimer and the last one to the unreacted Hsp90-S260C-723 homodimer.

in substantial amounts, which might pave the way for future studies of this and other large molecules by NMR spectroscopy.

Discussion

In this study, we established EPL for a large dimeric protein, the molecular chaperone Hsp90. The crucial step to significantly improve the yield was restriction of the flexible charged segment between the N- and C-terminal reactants, generating Hsp90-Δlinker. We conclude that conformational dynamics associated with the unstructured flexible linker caused lower EPL yields for the longer fragment.

EPL requires usually high concentration of the reactants, in the millimolar range, to improve the ligation yields (Muralidharan and Muir, 2006). However, excellent ligation yields have been observed in some special cases using significantly lower concentrations. In case of the bacterial sigma factor, the two peptide fragments were found to interact non-covalently, making the ligation reaction a unimolecular process (Camarero *et al.*, 2001). In the concentration range tested, from 50 to 160 μM of Hsp90-K233C-D723, we have not observed a systematic increase in the reaction yield. We concluded that in this case other factors were limiting.

We have tested several conditions to optimise the EPL reaction efficiency for Hsp90. Some groups report the pH dependence of N-terminal cleavage and show it to be inefficient below pH 6.5 with optimal results obtained at pH 7.0 or 7.5 (Mills and Perler, 2005). In certain cases, the yield was shown to be lowest at pH 6.0 and increased with increasing solution pH, reaching a maximum at pH 9.0 (Hauser and

Ryan, 2007). Another study shows that both splicing and cleavage of intein was slow at pH 8.3 and increased with decreasing pH, with the maximum yield at pH 7.5 (Wu *et al.*, 2002). On the other hand, the amide–thioester equilibrium at the last step of the ligation favours amide bond formation at neutral pH (Shao and Paulus, 1997). Those data indicate that different steps of EPL have different pH optima and the optimal pH might vary in the range of pH 6.0–9.0 for different reactions. In our case, we did not observe significant influence of the pH changes between 7.0 and 8.5.

The EPL reaction requires a complex protocol that consists of several steps to achieve the final ligation product. The accumulation factor resulting from this multi-step procedure typically results in low yields of the ligation product (Zhao *et al.*, 2008). We showed that the presence of a flexible charged linker in between at the reaction site decreased the efficiency of the EPL reaction for Hsp90, either due to the conformational flexibility or its uncommon highly positively charged amino acid composition. The application of the EPL on multimeric proteins to create segmental isotope-labelled domains can be used to define inter-domain interactions or study conformational changes upon ligand binding. Altogether, the EPL reaction is suitable for a homodimeric protein such as Hsp90, although not for all fragment boundaries. An up-scaling of the reaction and application to segmental isotope labelling for subsequent NMR analysis seems possible, but requires a significant amount of labelled media.

Materials and methods

Expression and purification of Hsp90-P1-E232, K233C-D723 and S260C-D723

Hsp90-P1-E232 cloned into a pTXB1 vector was expressed in *Escherichia coli* Rosetta 2 cells in M9 medium. Hsp90-P1-E232 was purified on a Q sepharose anion exchange column equilibrated in 25 mM phosphate buffer (pH 7.2).

Hsp90-K233C-D723 and Hsp90-S260C-D723 were expressed in pET23a(+) in *E.coli* Rosetta 2 cells in Luria-Bertani broth (LB) medium. Both were passed over a Q sepharose anion exchange column equilibrated in 25 mM phosphate buffer (pH 7.2) and subsequently purified on Ni-NTA-agarose resin (Qiagen). The protein was cleaved with Factor-Xa on the resin, overnight at 4°C, in Factor-Xa cleavage buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$ and 5 mM β-mercaptoethanol). The cleaved protein was then purified on a Poros 20 HQ anion exchange column equilibrated in 20 mM HEPES, pH 8.0.

Hsp90-S260-M552 was cloned into a pTXB1 vector with N-terminal hexa-His tag. Hsp90-S260-M552 was purified on a Chitin column and it was eluted with 10 mM DTT from Chitin column. After buffer exchange on Vivaspin column (10 kDa cut-off, Sartorius Stedim biotech), it was purified on Ni-NTA agarose-resin and eluted as explained for other constructs.

The proteins were concentrated on a Vivaspin column (10 kDa cut-off, Sartorius Stedim biotech) from 50 to 160 μM. Simultaneously, the buffer was exchanged to chitin buffer (20 mM HEPES, pH 8, 500 mM NaCl, 0.1 mM EDTA, 1 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.10%; Triton X-100) with complete protease inhibitor (Roche). All protein concentrations were determined by ultraviolet (UV) absorption measurements at 280 nm using a Nanodrop ND-1000 spectrophotometer based on the molar extinction coefficient of each protein fragment.

Cloning, expression and purification of SUMO-tagged Hsp90-K233C-723

Hsp90-S260C-D723 and K233C-D723 were cloned into the V275-pSUMO vector using BsaI and NotI restriction sites. Because the Hsp90 DNA sequence contains an internal BsaI site, two-step cloning was performed making use of an internal HindIII restriction site. Polymerase chain reaction amplifications of Hsp90-K233-C-HindIII, S260C-HindIII and HindIII-723 were performed using pET23a(+) with full-length Hsp90 as template.

SUMO-tagged Hsp90-K233C-D723 was expressed in *E.coli* Rosetta 2 cells in LB or Terrific broth medium and purified on a Poros 20 MC column charged with nickel sulphate, equilibrated in 25 mM HEPES, pH 8.0, 300 mM NaCl and 2 mM β-mercaptoethanol. An imidazole gradient from 1 mM to 1 M was used for elution.

The buffer of the purified SUMO-Hsp90 K233C-D723 was exchanged on a Vivaspin column into SUMO cleavage buffer (25 mM HEPES, pH 8.0, 300 mM NaCl and 5 mM β-mercaptoethanol). The protein was concentrated to 2 g/l and 4 mg Ulp1, the SUMO hydrolase was added per mg Hsp90 fragment. The SUMO tag was cleaved overnight at 4°C. After cleavage, the reaction mixture was purified on a Poros 20 MC column as described above.

Intein-mediated protein ligation

The reaction was performed in chitin buffer. The pH of the buffer was kept at pH 8.0 otherwise indicated. To test the effect of pH, it was varied from 7.0 to 8.5 in different ligation experiments. Hsp90-P1-E232 with intein and CBD was loaded onto 1.2 ml of chitin beads (binding capacity 45 nmol of Hsp90 fragment per millilitre resin) (New England Biolabs) to saturate the column. Then the column was washed with 15 column volumes (CVs) and equilibrated with 5 CV in chitin buffer with 50 mM MESNA and protease inhibitor cocktail (Roche). Hsp90-K233C-D723 or Hsp90-S260C-D723 in the same buffer with concentration ranging from 50 to 160 μM was passed through the column three times. A total of 200 μl of C-terminal fragment was added to start the reaction. EPL was carried out at 4°C for up to 2 weeks under continuous rotation in a locked Econo-column (Biorad). The reaction mixture containing Hsp90-Δlinker was eluted from the chitin beads in chitin buffer containing complete protease inhibitor cocktail (Roche).

For reactions that were not sufficiently efficient to isolate the final product, relative efficiencies were estimated from SDS–PAGE. For the Hsp90-Δlinker construct, the reaction was sufficiently efficient to isolate the final product. The yield of the reaction was calculated based on determination of all soluble protein fragments based on UV absorption measurements at 280 nm. The amount of Hsp90-CBD fusion protein on the chitin beads was assessed based on the amount of protein maximally bound to the solid phase under saturating conditions. The specification of the column is 2 mg maltose binding protein-CBD fusion protein per

millilitre resin (New England Biolabs). For the smaller Hsp90-P1-E232-CBD fusion protein this corresponds to 1.7 mg protein per millilitre resin.

Purification of the EPL product

The EPL product eluted from chitin beads were diluted 1:10 in heparin buffer (20 mM HEPES, pH 8.0, 1 mM TCEP and 0.5 mM $MgCl_2$). Purification was carried out on a HiTrap heparin column (GE Healthcare) using a salt gradient from 50 to 550 mM NaCl in heparin buffer.

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