CHEMBIOCHEM

Supporting Information

CHEMBIOCHEM

Supporting Information

for

Segmental Isotopic Labelling of a Multidomain Protein by Protein Ligation using Protein *Trans*-Splicing

Mikko Muona, A. Sesilja Aranko, and Hideo Iwai*

Construction of plasmids

The gene of nSH3(131-205) from c-CRK II was amplified from plasmid pAT044[1] (a kind gift from Dr. Patrik Forrer) by the two oligo nucleotides: #SK202 TCATATGCAG-GAGGAGGCAGAGTATGTG, and #SK201, TCGGATCCCTGGTTACCTCCAATCAG. The PCR product was ligated into a vector pHYRSF49-36 after digestion with *Nde*I and *Bam*HI, which resulted in pMMRSF17 for the expression of H_6-Smt3-nSH3-*Npu*DnaE-Int_{N123}. pHYRSF49-36 was derived from pHYRSF1 containing the fragment from pSKDuet1 digested with *Nco*I and *Hind*III and a mutation of *Spe*I site after the N-terminal His-tag. The gene of yeast ubiquitin-like protein, Smt3 amplified from *S. cerevisiae* genome was ligated between *Spe*I and *Nde*I sites. Furthermore, the gene of *Npu*DnaE-Int_{N102} was replaced with that of *Npu*DnaE-Int_{N123} using *Bam*HI and *Hind*III sites, which finally resulted in pHYRSF49-36.

The gene of cSH3(213-304) of c-CRK II was amplified from pAT044 and ligated into pHYBAD44(pMMBAD16 for the expression of Int_{C15}-cSH3) after the digestion with *Kpn*I and *Hind*III. The gene of *Npu*DnaE-Int_{C15} was amplified together with the gene of GB1 from pSKDuet16 by the two oligo nucleotides: #HK146: TACATATGGACCA-TAATTTTGCACTC and #T7-rev: GCTAGTTATTGCTCAGCGG and then ligated into

pSKBAD2 after digestion with *Nde*I and *Hind*III, resulting in pHYBAD44. All the plasmids were sequenced to confirm the correct DNA sequences. The gene of Int_{C15}-cSH3 was transferred to pRSF-1b vector (Invitrogen) in order to add an N-terminal His-tag, resulting in pMMRSF1-16.

Expression of fully ^{15}N-labelled ligated nSH3-cSH3

E.coli ER2566 cells harbouring the two plasmids encoding His-tagged Smt3-nSH3-Int_{N123} (pMMRSF17) and Int_{C15}-cSH3 (pMMBAD16) were initially grown at 37 ºC in 3 x 650 mL M9 medium supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin and $^{15}NH_4Cl$ (0.5 g /L) and D-glucose (2.0 g/L containging 10% $^{13}C_6$ D-glucose) as the sole nitrogen and carbon sources. Int_{C15}-cSH3 was first induced at a final concentration of 0.14 % L-arabinose for 0.5 h when the cell density reached OD_{600} = 0.5-0.6, followed by induction of the N-terminal fragment of His-tagged Smt3-nSH3-Int_{N123} with a final concentration of 1.0 mM IPTG. The cells were induced for in total 6 h at 37 ºC from the fist induction with L-arabinose. The cells were harvested and frozen at -80 ºC for further purification.

In vivo segmental isotopic labelling

E.coli ER2566 cells transformed with the two plasmids for His-tagged Smt3-nSH3-Int_{N123} (pMMRSF17) and Int_{C15}-cSH3 (pMMBAD16) were grown at 37 ºC in 3×750 mL M9 medium supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin and $^{15}NH_4Cl$ (0.5 g /L) as the sole nitrogen. Int_{C15}-cSH3 was first induced at a final concentration of 0.2 % L-arabinose for 3.0 h when the cell density reached OD_{600} = 0.5-0.8. Subsequently, the cells were spun down for 10 min at 850 *g* with SLA-3000 rotor and briefly washed with 200 mL LB medium containing 0.5 mM IPTG, 100 µg/mL ampicillin, and 25 µg/mL kanamycin. After the washing step followed by centrifugation at 800 *g* for 10 min at 10 ºC, the cells were resuspended in 3 x 600 mL LB medium containing 0.5 mM IPTG, 100 µg/mL ampicillin, and 25 µg/mL kanamycin. Expression of the N-terminal fragment of His-tagged Smt3-nSH3-Int_{N123} was induced for another 4.5 h at 37 ºC in the presence of a final concentration of 0.5 mM IPTG. The cells were harvested and frozen at -80 ºC for further purification.

In vitro protein ligation of nSH3 and cSH3

His-tagged Smt3-nSH3-Int_{N123} (pMMRSF17) was overexpressed by induction with 0.5 mM IPTG in 2 L of LB Medium supplemented with 50 µg/mL kanamycin and purified

by IMAC, which was followed by dialysis against PBS, pH 7.4. His-tagged Smt3 was digested with Ulp1 from *S. cerevisiae*[2] and removed by IMAC. The protein nSH3-Int_{N123} was dialyzed against 10 mM Tris, 500 mM NaCl, pH 7.0. His-tagged Int_{C15}-cSH3 encoded in the plasmid pMMRSF1-16 were overexpressed with 0.04% arabinose in 2 L of LB medium supplemented with 100 μg/mL ampicillin and purified by IMAC followed by dialysis against 10 mM Tris, 500 mM NaCl, pH 7.0. In vitro protein ligation was initiated by mixing the two purified protein in 10 mM Tris, 500 mM NaCl, pH 7.0 at the presence of 0.5 mM tris(2-carboxyethyl)phosphine (TCEP). The reaction mixture was agitated at 25 °C for overnight. Small amounts of the sample were taken during the incubation for SDS-PAGE analysis.

In vitro protein ligation of [^{15}N]-nSH3 and cSH3

His-tagged Smt3-nSH3-Int_{N123} (pMMRSF17) was overexpressed in *E.coli* ER2566 by the induction with 0.5 mM IPTG in 2 L of M9 medium containing $^{15}NH_4Cl$ as a sole nitrogen source supplemented with 50 μg/mL kanamycin and purified by IMAC. The protein [^{15}N]-his-smt3-nSH3-Int_{N123} was dialyzed against 10 mM Tris, 500 mM NaCl, pH 7.0. His-tagged Int_{C15}-cSH3 encoded in the plasmid pMMRSF1-16 were overexpressed with 0.04% arabinose in 2 L of LB medium supplemented with 100 μg/mL ampicillin and purified by IMAC followed by dialysis against 10 mM Tris, 500 mM NaCl, pH 7.0. In vitro protein ligation was initiated by mixing the two purified protein in an approximately 30 mL volume at the presence of 0.5 mM tris(2-carboxyethyl)phosphine (TCEP). The reaction mixture was agitated at 4 ºC for overnight. The ligated product was purified by IMAC and digested with Ulp1 from *S. cerevisiae*[2]. The fusion protein smt3 and Ulp1 protease were removed by IMAC. The flow through fractions were dialyzed against 10 mM Na phosphate, pH 8.0 and purified by MonoQ with a linear gradient of NaCl.

Purification of nSH3-cSH3

The harvested cells were suspended with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0 and lysed by ultra-sonication. The cell debris was removed from the protein solution by centrifugation for 45 min at 45 000 *g*. For Ion Metal Affinity Chromatography (IMAC), the entire supernatant was loaded on a Hitrap Chelating HP 5 mL (GE Healthcare) equilibrated with lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0). The column was washed with 50 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole, pH 8.0. The bound protein was eluted from the column with a liner

gradient to 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0. The fractions containing the fusion protein is dialyzed against PBS, pH7.4 overnight and digested with His-tagged Ulp1 for 2 h at room temperature. The undigested fusion protein, His-tagged Ulp1, and cleaved His-tagged Smt3 proteins was removed by passing through Hitrap Chelating HP 5mL (GE Healthcare). The ligated nSH3-cSH3 was eluted with 50 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole, pH 8.0. The nSH3-cSH3 fractions are dialyzed against 10 mM sodium phosphate pH 8.0 and further purified with MonoQ 5/50 GL (GE Healthcare).

NMR spectroscopy

All NMR measurements were performed on a Varian Innova 600 equipped with a triple resonance cryogenic probehead. The spectra were recorded with ca 0.1-0.4 mM samples at 25 °C in 250 µL volumes. A total of 256 (w_1) x 638 (w_2) data points with $t_{1,max}$ = 119.0 ms, $t_{2,max}$= 84.8 ms were recorded for the 2D [^{1}H, ^{15}N]-TROSY experiments. For data processing and spectra analysis, the programs PROSA[3], XEASY[4] were used.

References

[1] Forrer, P. Jaussi, R. *Gene* **1998**, *224*, 45-52.

[2] Mossessova, E. Lima, C.D. *Mol. Cell* **2000**, *5*, 865-876.

[3] Güntert,P., Dotsch,V., Wider,G., Wüthrich,K. *J. Biomol. NMR* **1992**, 2, 619-629.

[4] Bartels, C., Xia, T.H., Billeter, M., Guntert, P., Wüthrich,K. *J. Biomol. NMR* **1995**; 6, 1-10.