Mutual synergistic protein folding in split intein

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Synopsis

Inteins are intervening protein sequences that undergo self-excision from a precursor protein with the concomitant ligation of the flanking polypeptides. Split inteins are expressed in two separated halves, and the recognition and association of two halves are the first crucial step for initiating trans-splicing. In the present study, we carried out the structural and thermodynamic analysis on the interaction of two halves of DnaE split intein from Synechocystis sp. PCC6803. Both isolated halves (hI and Ic) are disordered and undergo conformational transition from disorder to order upon association. ITC (isothermal titration calorimetry) reveals that the highly favourable enthalpy change drives the association of the two halves, overcoming the unfavourable entropy change. The high flexibility of two fragments and the marked thermodynamic preference provide a robust association for the formation of the well-folded hI/Ic complex, which is the basis for reconstituting the trans-splicing activity of DnaE split intein.

Key words: disordered protein, intein, NMR spectroscopy, protein folding.

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INTRODUCTION

Protein splicing is a post-translational process in which intervening proteins, termed inteins, are self-excised from the precursor proteins, while the two flanking polypeptides (exteins) are seamlessly ligated together with a normal peptide bond [1]. Two types of protein splicing occur naturally: cis-splicing with inteins having continuous sequences or trans-splicing with inteins having split sequences. In comparison with cis-splicing, trans-splicing has one additional binding step, in which two fragments of split inteins recognize and associate with each other to reconstitute splicing activity, followed by the four steps in protein splicing mechanism (Figure 1) [2]. Protein trans-splicing can be easily controlled because each individual fragment can be prepared separately in an inactive form, and protein splicing occurs only after the reconstitution of the two complementary fragments. Thus, split inteins have been used for various applications in protein engineering, such as segmental isotopic labelling, protein semi-synthesis and detection of protein–protein interactions [3–5].

The DnaE intein from the split dnaE gene of Synechocystis sp. PCC6803 is a naturally occurring split intein, which has trans-splicing activity both in vivo and in vitro [6,7]. The crystal structure of an artificially fused continuous DnaE intein has been reported to adopt a horseshoe-like three-dimensional structure, termed the HINT (hedgehog/intein) fold, although the attempt to co-crystallize the two fragments of DnaE split intein failed [8]. A fast association rate (2.8×10^7 M^-1·s^-1) with high affinity (Kd~36 nM) of DnaE split intein was determined by FRET (fluorescence resonance energy transfer) [9]. Furthermore, the electrostatic interactions between split intein halves have been postulated to play a critical role during the binding process of the split DnaE intein [9–11]. Although the protein splicing mechanism has been extensively studied [11–14], the recognition of two fragments of split inteins, which determines the reconstitution of trans-splicing activity, is still poorly understood at the atomic level. All reported structures of inteins, including artificially fused split inteins, share a common HINT fold. However, the artificially split protein fragments from continuous inteins are prone to misfold or aggregate in individual expression in Escherichia coli [15]. The solution behaviours of individual

Abbreviations used: CDF, cumulative distribution function; CH plot, charge hydropathy plot; DLS, dynamic light scattering; DTT, dithiothreitol; GB1, B1 domain of protein G; GdmCl, guanidinium chloride; HINT, hedgehog/intein; HSQC, heteronuclear single quantum correlation; IDP, intrinsically disordered protein; ITC, isothermal titration calorimetry; IPTG, isopropyl β-D-thiogalactoside; KSI, ketosteroid isomerase; LB, Luria–Bertani; Ni-NTA, Ni2+–nitrilotriacetate; RBS, ribosome-binding site; SET, solubility enhancement tag; TEV, tobacco etch virus.

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halves from the naturally occurred split intein and their recognition have not been carefully studied.

To gain insight into the recognition of split inteins, we cloned and purified two halves of DnaE split intein, IA containing three intein residues and IC without intein. Our results show that, in the absence of the complementary partner, both IA and IC are unstructured under physiological conditions. The conformational changes occur upon association in an enthalpy-driven process. The inherent flexibilities with abundance of polar contacts provide an efficient approach to reconstitute protein trans-splicing.

EXPERIMENTAL

Plasmids

The gene encoding IA (amino acids 1–123) was amplified by PCR from pMEB10 with the following primers: forward, 5'-TACTTCCAATCCAATGCGTGCCTGTCTTT-3' and reverse, 5'-TTATCCACTTCCAATGCTATTTAATTGTCCCAGCGT-3'. The PCR product was cloned into a modified pMCSG7 vector with N-terminal His6-GB1 (the B1 domain of protein G) tag as the template described above using forward (5'-CGAcagatgctgGTTA-3') and reverse (5'- GCCgtcgagCTA-3') oligonucleotide primers. The PCR product was cloned into the NdeI–EcoRI-digested pET-21b(+) vector (Novagen). This construct is called pEGN for the expression of sole IA without the SET (Figure 2B). The co-expression plasmid containing genes encoding His6-IA and IC was constructed by two-step PCR using pEGN described above according to the previously published method [16]. IC and a second RBS (ribosome-binding site) were amplified with the IC PCR products as the template described above using forward (5'-TTAACCTTAAGAGAGATATCATATCTGGTAAGAGGCAGTGC-3', with RBS underlined) and reverse (5'-CCGctcgagCTAAGGCAGATAGC-3', containing an XhoI site denoted in lower case letters) oligonucleotide primers. The second PCR was done with the first PCR product as the template using the forward (5'-CGgaattcCTA-3') and a second RBS (ligation-independent cloning) method. This construct is named pENC for the co-expression of IA/IC complex (Figure 2C). The gene for IC (2–36 amino acid) was amplified from pKEC3 via PCR using forward (5'-CGGaattcCTA-3') and reverse (5'-CGAcagatgctgGTTA-3') oligonucleotide primers. The PCR product was cloned into the NdeI–EcoRI-digested pET-21b(+) vector (Novagen), a vector for high-level expression of peptide with an N-terminal KSI (ketosteroid isomerase) tag. This construct is named pEKC for preparation of the C-terminal fragment of DnaE split intein (Figure 2D). All clones were verified by DNA sequencing.

Protein expression and purification

N-terminal fragment (IA)

Plasmid pEGN was introduced into E. coli ER2566 cells. The cells were grown in 1 litre of LB (Luria–Bertani) medium with 100 mg/ml ampicillin at 37 °C until the cells reached a OD₆₀₀ of 0.6–0.8, then the protein expression was induced by 0.5 mM
IPTG (isopropyl β-D-thiogalactoside) at 16°C for 16 h. The cells were harvested by centrifugation at 4000 g for 20 min and resuspended in buffer A (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 5 mM 2-mercaptoethanol and 10 mM imidazole), and sonicated in an ice bath. Cell lysates were centrifuged at 22 324 g for 30 min at 4°C, and then the supernatant was loaded on to a column of Ni-NTA (Ni²⁺-nitrilotriacetate) resin (Qiagen) pre-equilibrated with buffer A. The fusion protein was eluted by buffer B (buffer A with 250 mM imidazole). The protein was concentrated and the imidazole was removed by ultrafiltration using a membrane with a 10-kDa molecular mass cut-off (Amicon). The His6-GB1 tag was digested using TEV (tobacco etch virus) protease at 16°C overnight in 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA and 1 mM DTT (dithiothreitol). After removal of EDTA and DTT by ultrafiltration with a 3-kDa molecular mass cut-off, the enzyme-digested products were purified again using Ni-NTA resin to remove His6-GB1 tag, TEV protease and the undigested proteins. The IC (containing three additional N-terminal residues (Ser-Asn-Ala) from TEV digestion) in the flow-through fraction was purified further by a Superdex-75 gel filtration column 16/60 (GE Healthcare) with eluent containing 20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA and 5 mM 2-mercaptoethanol. The molecular mass of IC was confirmed by SDS/PAGE (15 % gel) and MS. The concentration of IC was determined by UV absorption at 280 nm with the theoretical molar absorption coefficient (ε = 12950 M⁻¹·cm⁻¹).

C-terminal fragment (IC)
Plasmid pEKC was transformed into E. coli BL21 (DE3). The cells were grown in 1 litre of LB medium with 100 µg/ml ampicillin at 37°C until the cells reached a D600 of 0.6–0.8, then the protein expression was induced by 0.8 mM IPTG at 37°C for 5 h. IC was prepared using method given in the literature [17] with the following modifications. After the CNBr digestion, the pH of the solution diluted 1:4 (v/v) with MiniQ water was adjusted to approximately 4.5 with ammonia to yield white precipitates containing KSI and IC. IC was released from the precipitate by MiniQ water treatment and further purified by RP-HPLC (reverse-phase HPLC) using a C18 kromasil column (10 mm × 250 mm, 5 µm) at a flow rate of 3 ml/min with two-step linear gradient: 0–5 min: 10–30 % eluent B; 5–20 min: 30–100 % eluent B, where eluent A is 0.1 % TFA in Mini-Q water and eluent B is methanol. The purified IC was freeze-dried. The molecular mass of IC (containing one additional N-terminal leucine residue from CNBr cleavage) was confirmed by MS. The concentration of IC was determined by the BCA (bicinchoninic acid) assay.

Co-expression system
The co-expression plasmid pENC was transformed into ER2566 cells. The cells were grown in 1 litre of LB medium with 100 µg/ml ampicillin at 37°C. Protein expression was induced when cells reached a D600 of 0.6–0.8 with 0.1 mM IPTG at 37°C for 3 h. Cells harvested from cultures were resuspended in buffer A and sonicated in an ice bath. The ISN/IC complex was isolated from the supernatant on an Ni-NTA column and further purified by a Superdex-75 gel filtration column 16/60 (GE Healthcare) as described for the purification of ISN. The concentration of the ISN/IC complex was also determined by UV absorption at 280 nm with the theoretical molar absorption coefficient (ε = 12950 M⁻¹·cm⁻¹).

N-terminal fragment without SETs
For expression of sole ISN, plasmid pEN was transformed into E. coli ER2566. The cells were grown in 4 ml LB medium with 100 µg/ml ampicillin at 37°C. Protein expression was induced when cells reached a D600 of 0.6–0.8 with 0.1 mM IPTG at 37°C for 3 h. The cells were harvested using a 1.5 ml Eppendorf tube and resuspended using buffer A, and then sonicated in an ice bath. Cell lysates were centrifuged at 22 324 g for 10 min at 4°C and the inclusion bodies were dissolved in 8 M urea. Both supernatant and inclusion bodies were analysed by gel electrophoresis. In order to compare the influence of IC on the expression of ISN, both uninduced and induced cells harbouring the co-expression plasmid pENC were treated in the same manner as cells harbouring pEN.

15N-Labelled proteins
The uniformly 15N-labelled samples were prepared in the same procedure, except for replacing the LB medium with the M9 medium supplemented with 15NH₄Cl as the sole nitrogen source. The samples of ISN and the ISN/IC complex were concentrated by ultrafiltration and the buffer was exchanged to NMR buffer (100 mM NaCl, 3 mM DTT and 20 mM sodium phosphate buffer at pH 7.0). The freeze-dried IC was dissolved directly in NMR buffer.

CD spectroscopy
Far-UV CD spectra were obtained with a Jasco J-810 apparatus equipped with a temperature-controlled water bath using quartz cuvette with a 1.0 mm path length at 25°C from 190 to 260 nm in 50 mM sodium phosphate buffer (pH 7.4). Spectra were recorded with 15 µM ISN with buffer solution as the blank. All measurements were repeated three times. The protein denaturation experiments were performed by adding 0–6 M GdmCl (guanidinium chloride; 1 M increments) to protein samples. CD spectra of the thermal denaturation experiments were recorded at 25, 55, 75 and 95°C respectively. The sample was allowed to stand for 5 min at each temperature prior to the measurements.

Gel-filtration chromatography
Gel filtration was performed on an AKTA purifier liquid chromatography system using a Superdex-75 10/300 GL pre-packed column (GE Healthcare), which was pre-equilibrated with 20 mM Tris/HCl containing 100 mM NaCl (pH 8.0). The column was calibrated with RNase A (13.7 kDa, 16.4 Å; where 1 Å = 0.1 nm), chymotrypsinogen A (25.7 kDa, 20.9 Å), ovalbumin (44 kDa, 30.5 Å) and BSA (66.2 kDa, 35.5 Å). The results were analysed according to the method described previously [18].
RESULTS AND DISCUSSION

Expression of \( I_N \) and \( I_C \)

To study the interaction of two DnaE split intein fragments (\( I_N \) and \( I_C \)) and the properties of these fragments in solution, we first constructed a plasmid to produce \( I_N \) with an N-terminal His\(_6\) tag, but this fusion protein was expressed mainly in insoluble inclusion bodies at 16°C in \( E. \ coli \). To improve high-level expression of soluble \( I_N \), we next constructed a plasmid to produce \( I_C \) with an N-terminal His-tagged GB1 (6.2 kDa) as a SET and a TEV protease recognition site between GB1 and \( I_N \). The fusion protein (His\(_6\)-GB1-\( I_N \)) was purified by Ni-NTA affinity chromatography from supernatant and the fusion tag was removed by digestion with TEV protease. After protease digestion, \( I_N \) was purified further by Ni-NTA affinity chromatography, followed by gel filtration. The purified \( I_N \) was readily degraded at room temperature (25°C) by residual protease contaminants in the protein preparation. The degradation can be effectively inhibited with 2 mM protease inhibitor PMSF (results not shown). Therefore freshly prepared \( I_N \) was used in the present study.

\( I_C \) was prepared using the pET-31b(+) vector (Novagen) for the high-level expression of peptide. The protein was expressed with an insoluble expression tag (KSI tag) at the N-terminus in order to protect the peptide from proteolytic degradation in vivo. The KSI-\( I_C \) fusion was extracted from inclusion bodies using 8 M urea. The fusion protein was precipitated after removing urea by dialysis against ultra-pure water, and then freeze-dried. The KSI tag was removed by CNBr digestion in 88% formic acid. \( I_C \) was purified using RP-HPLC. The identities of both \( I_N \) and \( I_C \) were confirmed by MS.

Isolated fragments of split intein are disordered

Based on the intein structures solved so far, all inteins possess the \( \beta \)-rich HINT fold, which brings the N- and C-terminal splicing junctions close in space. Such a three-dimensional arrangement must play a crucial role in protein splicing. CD spectroscopy is the most commonly used method to determine the extent of secondary structure with the spectrosopic signatures of the \( \alpha \)-helices (negative bands at 208 and 222 nm) and \( \beta \)-sheets (a negative minimum at 218 nm and a positive maximum at 195 nm), whereas the random coils show a minimum near 198 nm. CD spectra showed that \( I_N \) possesses a nearly unstructured conformation, characterized by a single negative minimum at 203 nm (Figure 3A, black curve). As the artificially fused Ssp DnaE intein showed a well-ordered structure, this observation suggests that the short peptide sequence of \( I_C \) plays an important role in assisting the folding of \( I_N \). Meanwhile, the shoulder approximately 220–230 nm in CD spectra of \( I_N \) indicates that this disordered protein contains some residual secondary structure [21]. The chemical denaturation experiments confirmed the presence of the residual secondary structure, which were gradually lost with the addition of GdmCl (Figure 3B). It has been proposed that the residual structure in unstructured proteins or protein domains serves as a primary contact site and guides correct protein

Bioinformatic analysis

The amino acid composition of \( I_N \) was analysed using the composition profiler software [19], which includes the Disprot 3.4 and PDB_Select_25 datasets. The disorder prediction of \( I_N \) was performed using PONDR® software with VL-XT algorithm (http://www.pondr.com/), and then CDF (cumulative distribution function) analysis of the output of PONDR® was performed to provide the distribution of prediction scores and a linear boundary for distinguishing ordered and disordered proteins. The CH plot (charge hydropathy plot) was also done using PONDR®.

NMR spectroscopy

All \(^{1}H\)-\(^{15}N\) HSQC (heteronuclear single quantum correlation) spectra were recorded at 298 K on 500 MHz Bruker spectrometer equipped with a cryoprobe. All uniformly \(^{15}N\)-labelled protein samples were prepared in NMR buffer (100 mM NaCl, 3 mM DTT, 20 mM sodium phosphate buffer at pH 7.0) with 10% \(^{2}H_{2}O\). Spectra were processed and analysed using NMR Pipe [20] and Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, CA, U.S.A.).

Limited proteolysis

Limited proteolysis was carried out on \( I_N \) and the co-expressed \( I_N/I_C \) complex at 25°C with trypsin in 20 mM Tris/HCl, 1 mM DTT, 0.5 mM EDTA and 100 mM NaCl (pH 8.0). The enzyme to substrate ratio was 1:1000 (w/w). Aliquots were taken from the proteolysis solution at the various reaction times (0, 2, 4, 8, 16, 32 and 64 min), and the reaction was stopped by adding SDS loading buffer and boiling for 5 min. Cleavage products were resolved by Tris-tricine gel electrophoresis (15% gel) and visualized by staining with Coomassie Brilliant Blue R250.

ITC (isothermal titration calorimetry)

ITC experiments were performed on a Microcal ITC 200 (GE Healthcare) by titrating 150 \( \mu \)M \( I_C \) into 20 \( \mu \)M \( I_N \) at 25°C in 20 mM sodium phosphate buffer (pH 7.4) and 100 mM NaCl. The heat of dilution was measured by titrating \( I_C \) into blank buffer, and the net binding heat was obtained by subtracting the dilution heat from the apparent reaction heat. Data were analysed using Origin ITC software.
folding by limiting the conformational space in protein–protein recognition [22]. Therefore the residual structure in IN can be helpful in the association of two halves of DnaE split intein.

To test the stability of the residual secondary structure in IN, the CD spectra were recorded from 25 to 95 °C. Interestingly, the CD spectra of IN exhibit very little change over the temperature range, in which the ellipticity (mDeg) at 222 nm slightly decreases with temperature increase (Figure 3A). The temperature independent CD spectra are consistent with IDPs (intrinsically disordered proteins) [23]. Disordered proteins typically exhibit abnormally larger hydrodynamic radius (Stokes radius) than the globular proteins with the same molecular mass [24]. To test whether this is true for IN, the hydrodynamic radius of IN was first measured by gel filtration. The results showed that IN (14.2 kDa) has a larger hydrodynamic radius (24 Å) than the corresponding molecular mass of globular protein (19 Å), calculated by the equation: log(RNS) = −0.204 + 0.357 × log M, where RNS is the expected Stokes radius of globular proteins [23]) (Figure 3C). Meanwhile, the hydrodynamic radius of IN determined by DLS gave a very similar value (22 Å) to the gel filtration result (Figure 3D). Furthermore, gel electrophoresis showed that the unusual solubility during boiling protects IN from insoluble aggregates, which also supports the natively unfolded state of IN (Figure 3E) [24].

For both IN and IC, two-dimensional ¹H-¹⁵N HSQC NMR spectra were recorded on the uniformly ¹⁵N-labelled protein samples. Results showed very poor dispersion for both IN (Figure 4A, blue) and IC (Figure 4B, blue) in the isolated form, which demonstrated the disordered state of these two fragments. Taken together, these results suggest that both IN and IC in separated form are in disordered state.

It has been reported that the IDPs typically have more disorder-promoting residues and less order-promoting residues, based on the bioinformatics analysis [25]. However, the amino acid
composition analysis showed that \( I_N \) does not belong to the class of IDPs compared with the Disprot 3.4 dataset (Figure 5A). This result suggested that \( I_N \) has the potential to adopt an ordered structure. The intrinsic disorder propensity of \( I_N \) was further analysed by a neural network-based predictor PONDR® (http://www.pondr.com/) using the VL-XT algorithm with default parameters. The CDF analysis of the output of PONDR® has been used to provide the distribution of prediction scores and a linear boundary for distinguishing ordered and disordered proteins [26]. The CDF curve showed that \( I_N \) is located in the range of ordered proteins (Figure 5B). In addition, the CH plot, a plot of the normalized Kyte–Doolittle hydropathy against the mean net charge to classify ordered and disordered proteins [27], also located \( I_N \) in the ordered region (Figure 5C). These theoretical predictions consistently indicate that \( I_N \) has strong potential to form an ordered structure, although it demonstrated a disordered state experimentally. Therefore these results suggest that the disordered \( I_N \) is ready to fold into a well-structured protein in the association with its complementary partner.

**Folding upon interaction**

Although both \( I_N \) and \( I_C \) exhibit unfolded states in the isolated form, the X-ray crystal structure indicates that the artificially fused DnaE intein possesses a HINT structure. This fold is shared by all reported structures of inteins, so that it is very likely also present in split inteins after the association of two fragments, although no structures of split intein complex have been reported. Disordered proteins are usually involved in folding upon binding [28]. To verify the conformational change of \( I_N \) and \( I_C \) after association, \(^{1}H^{15}N\) HSQC spectra were recorded on \(^{15}N\)-labelled \( I_N \) in the absence and presence of non-labelled \( I_C \). The spectrum of \( I_N \) shows a significant improvement in signal dispersion after adding \( I_C \) (Figure 4A, red). A similar dispersion improvement was also observed on the \(^{15}N\)-labelled \( I_C \) upon adding \( I_N \) (Figure 4B, red). This result clearly indicates the disorder-to-order transition of \( I_N \) upon binding to \( I_C \), and vice versa. Therefore, it can be concluded that the disordered structure of the isolated fragments of \( I_N \) and \( I_C \) is due to the split of the continuous DnaE intein, and the mutual synergistic folding occurs during the association of \( I_N \) and \( I_C \), which is a vital step for protein trans-splicing.

**Limited proteolysis**

It has been reported that the disordered proteins are more easily digested by proteases because the residues are largely exposed to solvent. On the contrary, ordered proteins are more resistant to proteases as the compact structures protect cleavage sites from protease access [29]. Therefore the limited proteolysis can be used to probe the conformational characteristics of proteins. Here, the protease sensitivity of \( I_N \) and \( I_N/ I_C \) protein complex were examined using trypsin. Tris-tricine gel electrophoresis (15 % gel) was used to monitor the time course of protease digestion. The results show that \( I_N \) is prone to digestion as an individual fragment (Figure 6, upper panel); however, the protein gained great protease resistance in the co-expressed binary complex (Figure 6, lower panel). This result further confirmed that the disordered protein of \( I_N \) forms a folded three-dimensional structure in the binary complex.

**\( I_C \) assists the folding of \( I_N \) in vivo**

We next examined the association of two halves of DnaE intein in vivo using a co-expression system. The plasmid carrying two different genes, encoding \( I_N \) and \( I_C \) with two RBSs, was constructed according to the approach reported previously [16]. By transformation of the plasmid into E. coli ER2566 strain, both \( I_N \) and \( I_C \) were co-expressed simultaneously. In contrast with the expression of \( I_N \) alone, which mainly yielded the protein in insoluble inclusion bodies in E. coli, the co-expression system produced the soluble \( I_N/ I_C \) complex in the supernatant (Figure 7A). The formation of insoluble \( I_N \) is likely attributed to the disordered nature of \( I_N \), whereas the co-expression system generated the well-structured protein complex in the supernatant. It is well known that the newly synthesized proteins can escape protein misfolding and aggregation by folding modulators, such as molecular chaperones [30–32]. Thus, the co-expression data demonstrate that the expressed \( I_C \) can associate with expressed \( I_N \) within E. coli and assist the folding of \( I_N \) in vivo.
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**Figure 5** Bioinformatic analysis of \( I_N \)

(A) Comparisons of amino acid compositions of \( I_N \) (white bars) and Disprot 3.4 (black bars) with PDB_Select_25 datasets using Composition Profiler. The values of amino acids given according to Vihinen’s flexibility scale represent enrichment (positive) or depletion (negative) relative to PDB_Select_25 dataset. \( I_N \) has less disorder-promoting amino acids but one residue (E, \( P < 0.05 \)), and more order promoting amino acids than Disprot 3.4 dataset. (B) CDF analysis of \( I_N \). The black line represents the boundary between order (above) and disorder (below). \( I_N \) located in the order region is shown as a grey curve. (C) The CH plot of \( I_N \). A linear boundary (solid) (\( R = 2.785(H) - 1.151 \)) separates the CH plot into disordered (above) and ordered (below) regions. \( I_N \) (■) is located at the ordered region.

**Figure 6** Limited proteolysis of purified \( I_N \) (upper panel) and \( I_N/I_C \) complex (lower panel) with trypsin.

Reaction time was labelled in figure. The enzyme to substrate ratio was 1:1000 (w/w). Cleavage products were resolved by 15% Tris-tricine gel electrophoresis and visualized by staining with Coomassie Brilliant Blue R250.

To characterize further the \( I_N/I_C \) complex, the co-expression product was isolated using Ni-NTA affinity chromatography, and further purified by gel-filtration chromatography. The collected fractions were subjected to Tricine-SDS/PAGE (15% gel) and two protein bands corresponding to \( I_N \) and \( I_C \) were observed (Figure 7B). This result indicates that the \( I_N/I_C \) complex is highly stable during the chromatography processes. To verify the conformation of the binary complex, the \( ^1H-^{15}N \) HSQC NMR spectrum was recorded on the \( ^{15}N \)-labelled sample from the co-expression system. The well-dispersed backbone amide resonances provide solid evidence that the \( I_N/I_C \) complex is well folded (Figure 7C). Gel filtration clearly showed that the folded \( I_N/I_C \) complex has a smaller Stokes radius than disordered \( I_N \) (Figure 7D). This result reveals that the two fragments of split inteins can recognize and associate with each other \textit{in vivo} to form a well-defined three-dimensional structure, which is essential for splicing activity. Similar results have also been observed in the split DnaE intein from \textit{Nostoc punctiforme} (results not shown).

**Thermodynamics of interaction**

The thermodynamic parameters of the interaction between the two fragments of split intein were quantitatively measured by ITC. The titration of \( I_C \) into \( I_N \) was an exothermic process, illustrated by negative peaks in Figure 8. A non-linear best-fit binding isotherm yielded a dissociation constant (\( K_d \)) of 33 nM (Figure 8). This datum agrees with the previously reported result from the...
Figure 7  Characterization of the \(h_N/\text{I}_C\) complex produced by co-expression

(A) Recombinant expression of \(h_N\) alone and co-expression of \(h_N\) with \(\text{I}_C\). The supernatant (S) and the pellet (P) from cell lysis were analysed by SDS/PAGE (15\% gel) and visualized by staining with Coomassie Brilliant Blue R250. The \(M_r\) of \(\text{I}_C\) is too small to be visualized on this gel. (B) The \(h_N/\text{I}_C\) complex purified from gel-filtration was resolved by 15\% Tris-tricine gel electrophoresis and visualized by staining with Coomassie Brilliant Blue R250. Lane 1 is co-expression products, lane 2 is protein markers. The bands corresponding to \(h_N\) and \(\text{I}_C\) are labelled in the Figure. (C) The \(^1\text{H}-^1\text{H}\) HSQC NMR spectrum of the uniformly \(^{15}\text{N}\)-labelled \(h_N/\text{I}_C\) complex from co-expression. (D) Gel filtration profiles of \(h_N\) alone and co-expression of \(h_N/\text{I}_C\) complex.

Figure 8  ITC profile of the titration of 150 \(\mu\text{M}~h_N\) into 20 \(\mu\text{M}~\text{I}_N\)

The top panel shows the raw data of the net binding isotherm, and the bottom panel shows the best fit curve (solid line) of the integrated enthalpy change (dot) to a one-site binding model using non-linear least-squares algorithm.

The interaction of extein-containing \(I_N\) and \(I_C\) fragments [GFP (green fluorescent protein) as N-extein and organic fluorophore Texas Red as C-extein] measured by fluorescence [9]. The thermodynamic parameters of enthalpy change \((\Delta H = -29.1\text{ kcal/mol})\) and entropy change \((\Delta S = -18.9\text{ kcal/mol})\) indicate that the binding is driven by enthalpy with a very unfavourable entropic cost. This phenomenon is known as the enthalpy–entropy compensation [33], which is often associated with folding upon binding. The favourable enthalpy change arises from a large number of polar contacts between \(I_N\) and \(I_C\). The unfavourable entropy change is likely due to the loss of conformational freedom caused by the disorder-to-order transition during the association [34].

It is well known that the folded structure is a prerequisite for most proteins to perform various biological functions. However, accumulated evidence in the last decade showed that disordered proteins are involved in many cellular processes, such as transcriptional regulation and signal transduction [35]. On the other hand, disordered proteins could undergo conformational transition from disorder to well-folded structure upon binding to their partners and then carry out their specific biological functions [29,36–38], and the availability of disordered proteins are tightly controlled in cells [39]. Protein recognition and association can be through different mechanisms, including lock-and-key, induced-fit, conformational-selection and fly-casting.
The fly-casting mechanism suggests that the disordered proteins provide a greater capture radius for speeding up association followed by protein unfolding [40]. This mechanism may be applicable to the association of two split intein halves, which results in efficient binding and reconstitution of protein splicing activity.

Conclusions

In summary, the results of the present study reveal that both halves of DnaE split intein are unfolded, and undergo large conformational changes upon the association of two fragments. The mutual synergistic folding is featured by the highly favourable binding enthalpy change and highly unfavourable entropy change. The disordered nature of split intein halves, and the polar interactions between the two halves pave the way for reconstituting protein trans-splicing activity. This work provides insights into protein recognition of two halves of split inteins, which is the first crucial step for initiating protein trans-splicing.

AUTHOR CONTRIBUTION

Yuchuan Zheng and Yangzhong Liu designed the research and co-wrote the paper, Yuchuan Zheng and Qin Wu performed the research, and Chunyu Wang and Min-qun Xu provided technical support.

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