Chapter 2

Chemically Induced Self-Assembly of Enzyme Nanorings

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Abstract

Continued exploration into the field of chemically induced dimerization (CID) has revealed a number of applications for its use in a broader context as a method of structural assembly (1–4). In particular, the use of CID technology to generate self-assembled (and selectively disassembled) protein toroids serves as a key advancement toward developing stable and controllable protein-based platforms. Such structures have broad application to the development of novel therapeutics, lab-on-a-chip technologies, and multi-enzyme assemblies (5, 6). This chapter describes a method of developing an enzymatically active protein nanostructure incorporating both a CID-based assembly region containing dihydrofolate reductase (DHFR) and an enzymatic region consisting of histidine triad nucleotide binding protein 1 (Hint1). Details of both the production and the characterization of this structure are provided.

Key words: Enzyme nanorings, DHFR, Hint1, nanostructures, self-assembly, chemically induced dimerization, bis-methotrexate, gel filtration, protein expression, protein purification.

1. Introduction

Chemically induced dimerization is the controlled dimerization of proteins via dimerizers. During the process of dimerization, the dimerizers assemble proteins into homospecific or heterospecific multivalent nanostructures. Mimicking the functions of biological inducers for protein dimerization to regulate cellular signaling pathway, CID systems have been developed as investigative tools for the selective activation of various cellular processes to control cell membrane receptor signal transduction and gene expression (7, 8). To demonstrate the competency of this technique, CIDs have also been exploited in the three-hybrid methodology for high-throughput bioscreening. Furthermore, the application of CID as a means to control the assembly of protein oligomers

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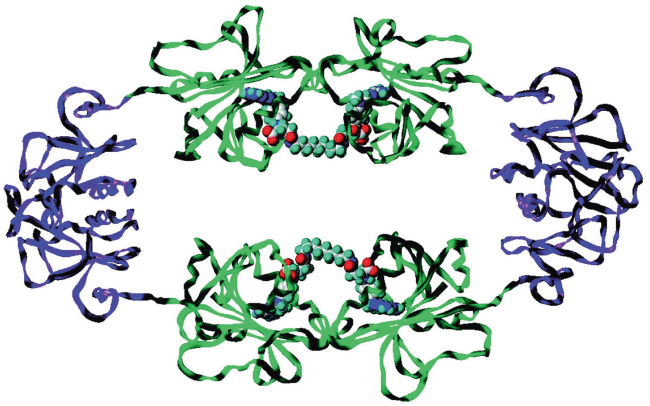

Fig. 2.1. Model of the DHFR–hHint1 dimeric nanoring. DHFR is shown in *green* and Hint1 in *purple*. The dimerizer is rendered in space-filling fashion. Figure reproduced from (5) with permission from the American Chemical Society.

offers an avenue to engineer protein-based materials and nanostructures.

The method of engineering enzyme nanorings described in this chapter is based on the CID system for the preparation of self-assembled protein macrocyclic oligomers of dihydrofolate reductase fusion proteins with a polypeptide linker of variable length ($DHFR_2$) by chemical induction with bis-methotrexate (bis-MTX) (2). The size of the nanorings is dependent on the length of the polypeptide linker between the two DHFRs. Human Hint1 is a highly stable homodimer and acts as a phosphoramidate and acyl-adenylate hydrolase. When incorporating enzyme human Hint1 between the two DHFRs, enzymatically active protein nanorings can be assembled (**Fig. 2.1**) (5). Similar to other $DHFR_2$-based protein nanorings, enzyme nanoring size is also dependent on the length and composition of the polypeptide linking the fusion proteins. The more general assembly of protein nanorings can be characterized by size-exclusion chromatography and their resulting enzymatic activity explored by typical activity assays.

2. Materials

2.1. General

1. Dithiothreitol (DTT) is a common reducing agent that should not be added to buffers until after pH adjustments are made. It will also become oxidized over time – do not use buffers containing DTT that is over ~1 week old.
2. The preparation of bis-methotrexate (**Fig. 2.2**) involves synthetic organic chemistry which is beyond the scope of this

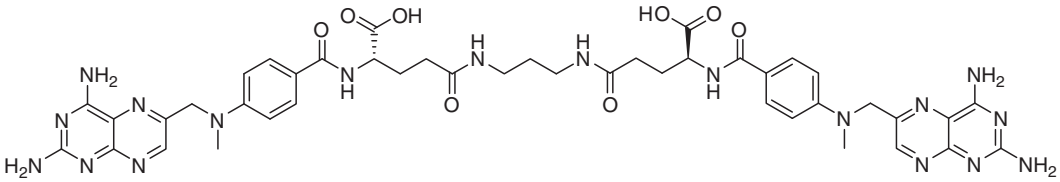

Fig. 2.2. Structure of bis-methotrexate. The preparation of the molecule is detailed in (8).

Fig. 2.3. The DHFR–hHint1 fusion protein. The DHFR proteins serve as the chemically induced dimerization domain while human Hint1 associates naturally and retains its enzymatic activity. The 3DH-GLE nomenclature stands for DHFR–hHint1 with a gly–leu–glu linker between the two proteins. Figure reproduced from (5) with permission from the American Chemical Society.

protocol, but a detailed description of its preparation has been published (9).

3. The construction of the DHFR–human Hint1 (hHint1) plasmid will not be covered here since the construction has been detailed elsewhere (10). This plasmid, or any suitable fusion of an enzyme gene to the DHFR gene, can be transformed into an expression cell line via any number of commercially available protocols (i.e., via Invitrogen- or Promega-competent cell kits).
4. As noted previously, this work utilizes a DHFR–hHint1 fusion protein (**Fig. 2.3**). Hint1 enzymatic activity is measured via the hydrolysis of a fluorogenic substrate, tryptamine 5′-adenosine phosphoramidate (TpAd), and this assay is described in detail elsewhere (11). However, the use of novel DHFR–enzyme constructs is encouraged, and enzymatic activity assays must be tailored to the new DHFR fusion partner.

2.2. DHFR Activity Assay

1. MTEN assay buffer: 50 mM 2-morpholinoethanesulfonic acid (MES), 25 mM Tris(hydroxymethyl)aminomethane–HCl, 25 mM ethanolamine, 100 mM NaCl, pH 7.0, 1 mM DTT. It may be helpful to make a 10× concentrated stock of this buffer and store at 4°C. In this case, add DTT only when diluting to the 1× assay buffer as to prevent premature oxidation of the DTT.
2. Dihydrofolate (DHF) is prepared fresh as described (12) and stored as slurried aliquots under argon at –80°C.

3. Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), should be dessicated and stored at –20°C.

2.3. Cell Growth and Lysis

1. Luria–Bertani powder (LB) is made by adding 20 g LB powder to 1 L of deionized water and autoclaving at 121°C for 20 min prior to use. LB broth may be stored at 4°C.
2. Ampicillin (Amp) is dissolved in deionized water at a stock concentration of 100 mg/mL, filter sterilized using a 0.22 μm syringe filter, stored at –20°C in aliquots, and added to LB broth and made to a final concentration of 100 μg/mL. Stock aliquots have a shelf life of ~2 weeks at −20°C and should only be thawed and re-frozen once.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is dissolved in deionized water at a stock concentration of 0.5 M, filter sterilized using a 0.22 μm syringe filter, and stored at −20°C. During protein expression, IPTG is added to the culture to a final concentration of 0.5 mM.
4. *E. coli* strain BB2: This strain is produced by disrupting the *E. coli hinT* gene in strain BW25113 as described (13). It is necessary to use hinT-deficient *E. coli* to avoid wild-type *E. coli* Hint1 contamination.
5. Lysis buffer A: 50 mM Tris–HCl, pH 8.0, 5 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mg/mL lysozyme, 50 μg/mL sodium azide, 1 mM DTT.
6. Lysis buffer B: 1.5 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 20 μg/mL DNase I, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT.
7. Dialysis buffer: 20 mM Tris–HCl, pH 7.0, 1 mM EDTA, 1 mM DTT.

2.4. Fusion Protein Purification

1. Methotrexate (MTX) agarose is stored at 4°C and must be protected from light.
2. Buffer A: 20 mM Tris–HCl, pH 7.0, 1 mM EDTA, 1 mM DTT.
3. Bio-Rad protein assay kit.
4. NuPAGE SDS-PAGE apparatus, 4–12% bis-Tris–HCl minigels, 4× NuPAGE loading buffer, 10× NuPAGE reducing agent, NuPAGE antioxidant, NuPAGE 20× MES-SDS running buffer (Invitrogen). Protein gel electrophoresis can be performed using the Invitrogen NuPAGE protocol.
5. Amicon stirred cell equipped with a YM-10 membrane or, alternatively, Amicon Centricon centrifugal filter devices may be used (with 10 kDa cutoff).
6. Diethylaminoethyl-cellulose DE52 (DEAE-DE52) ion exchange media.

7. Glycerol: Add 30 mL glycerol to 70 mL deionized water and autoclave at 121°C for 20 min to yield a sterile 30% solution of glycerol.
8. Trimethoprim.

2.5. Nanoring Assembly and Characterization

1. P500 buffer: 0.5 M NaCl, 50 mM KH_2PO_4, 1 mM EDTA, pH 7.0.
2. Superdex 200 10/300 GL Gel Filtration column (GE Healthcare) equipped on a Beckman System Gold HPLC with detection at 280 and 302 nm detecting the presence of protein and methotrexate, respectively.
3. Bis-methotrexate (bis-MTX or MTX_2C9) can be prepared as described above. It should be stored at –20°C and protected from light. Given these storage conditions, it is stable for greater than 1 year.

3. Methods

3.1. General

1. Transformation into the non-competent *E. coli* BB2 cell line can be achieved using the method of Hanahan (14). All purification steps are performed at 4°C unless otherwise noted.

3.2. DHFR Activity Assay

1. Add 1 mL MTEN buffer to an aliquot of DHF. The concentration of this stock solution can be determined by diluting 5 μL of the solution into 1 mL of MTEN and checking the A_{280} of the solution. The concentration (μM) is then equal to the absorbance multiplied by the dilution factor divided by 0.028 μM/L/cm, the extinction coefficient for DHF at 280 nm.
2. Dissolve 1–2 mg NADPH in 1 mL MTEN. The concentration of this stock solution can be determined as with DHF, using 0.0062 μM/L/cm as the extinction coefficient of NADPH at 340 nm.
3. Start the reaction by adding 50 μM DHF to a solution of DHFR (2–20 μL depending on estimated concentration) and 100 μM NADPH in MTEN assay buffer (1 mL final assay volume, *see* **Note 1**).
4. Monitor the conversion of NADPH to NADP+ in a UV spectrophotometer measuring absorbance at 340 nm over time. The slope of the resulting linear plot represents the activity in μmol/mg/min.

3.3. Cell Growth and Lysis

1. Starting from a plate of BB2 *E. coli* freshly transformed with the plasmid of interest, pick a single colony and inoculate 10 mL LB broth containing 100 μg/mL ampicillin. Grow the culture overnight at 37°C with shaking at 250 rpm.
2. Use the 10 mL culture to inoculate 1 L of LB broth containing 100 μg/mL ampicillin in a 2 L Erlenmeyer flask. Grow the culture at 37°C with shaking as before to an OD600 of 0.4 (*see* **Note 2**).
3. Add IPTG to a final concentration of 0.5 mM (*see* **Note 3**).
4. Incubate the culture for an additional 2.5 h at 37°C, then centrifuge the culture at 5,000×*g* for 15 min at 4°C. Cell pellets can be frozen and stored at –80°C for up to 1 week.
5. Resuspend the cell pellet in 2 mL of lysis buffer A per gram of cells and incubate at room temperature for 5 min. Digest DNA by adding 2 mL of lysis buffer B per gram of cells and incubating at room temperature for another 25 min.
6. Sonicate the resulting suspension for 12 rounds of 15 s, keeping the temperature of the lysate lower than 20°C. In this setup, a VibraCell VCX750 with temperature probe is used to automatically monitor the sonication process. Thirty-five percent of the maximum power of the unit is used during each pulse.
7. Centrifuge the lysate at 25,000×*g* for 45 min at 4°C to pellet the cell debris. Dialyze the supernatant overnight at 4°C against 2 L of dialysis buffer.

3.4. Fusion Protein Purification

1. Prepare the affinity column by adding 12.5 mL MTX agarose to a clean column, then equilibrating the media with 40 column volumes of buffer A.
2. Load the protein dialysate onto the MTX column at 1 mL/min.
3. Wash the protein with 40 column volumes of buffer A and 60 column volumes of buffer A containing 1 M NaCl, then elute the protein with 150 μM trimethoprim in buffer A. All wash and elution steps are performed at 1 mL/min and 9 mL fractions are collected.
4. Analyze 10 μL of each fraction via the Bio-Rad protein assay kit and assay fractions containing more than 0.1 mg/mL of protein via SDS-PAGE and the DHFR activity assay (*see* **Section 3.1**). Combine fractions containing the desired protein and remove the trimethoprim via buffer exchange (to buffer A) in a stirred Amicon chamber equipped with a YM-10 membrane. This buffer exchange step can also be utilized to concentrate the protein to ~2–3 mg/mL.

5. Prepare the DEAE column by pouring ~50–75 mL of media slurry into a clean column and equilibrate at 1.0 mL/min with 10 column volumes of buffer A.
6. Load the protein onto the DEAE column at 1.0 mL/min.
7. Elute the protein with a linear gradient between buffer A and 0.5 M NaCl in buffer A over 720 min. Collect 9 mL fractions at 1.0 mL/min and assay every other fraction for A_{280} to determine where the protein elutes. Analyze 10 μL of fractions containing significant absorbance (*see* **Note 4**) with the DHFR activity assay and SDS-PAGE.
8. Pool fractions containing the protein of interest and concentrate to 1–2 mg/mL using an Amicon chamber as before. Protein concentration can be determined using the Bio-Rad protein assay kit protocol.
9. Add glycerol to a final concentration of 15% and store the protein in aliquots at −80°C.

3.5. Nanoring Assembly and Gel Filtration

1. The concentration of bis-MTX can be determined spectrophotometrically by diluting 5 μL bis-MTX in 1 mL 0.1 M NaOH and reading the absorbance at 302 nm. This absorbance is multiplied by the dilution factor and divided by the extinction coefficient of bis-MTX, 0.0474 μM/L/cm.
2. To assemble the protein nanorings, add 1.1 moleq of bis-MTX to a sample of the fusion protein (typically 5–100 μM) in a final volume of 1 mL P500 buffer. Incubate at room temperature for 1 h (*see* **Note 5**).
3. Load 500 μL of the nanoring mixture on to the gel filtration column and elute at 0.5 mL/min with P500 buffer. A sample trace can be found in **Fig. 2.4** (*see* **Note 6**).

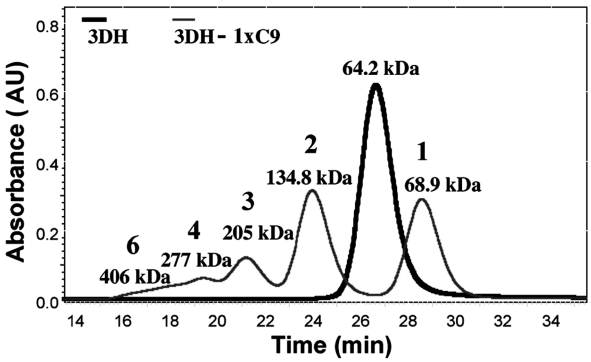

Fig. 2.4. Overlaid size-exclusion profiles for 3DH-GLE (*black*) and 3DH-GLE with 1 eq of bis-MTX added (*red*). *Numbers* above the peaks represent the number of fusion protein monomers (as shown in **Fig. 2.3**) in the ring. The peak eluting at ~29 min represents an intramolecular macrocycle (monomeric ring). Figure reproduced from (5) with permission from the American Chemical Society.

4. Further characterization of the nanorings may be accomplished via transmission electron microscopy, atomic force microscopy, light scattering, or any other desired means.

4. Notes

1. Mixing of reagents in the DHFR activity assay is best achieved by adding the MTEN, NADPH, then protein to a disposable cuvette, then using a piece of parafilm to cap off the vessel and inverting several times. After allowing the solution to incubate at room temperature for at least 1 min to allow NADPH loading onto DHFR, the DHF may be added. The consumption of NADPH begins immediately, so another round of mixing must be performed quickly before all the reagents are depleted. If zero activity is detected in assays where it is thought that the protein concentration is relatively high, it may help to reduce the amount of protein in the sample and try again. Additionally, it is common in samples containing a lot of protein to see the linear reaction rate slow (and become nonlinear) as all the DHF are reduced – velocity measurements should include only the linear region of the UV trace.
2. Induction times and temperatures may vary depending on the fusion protein chosen. Small-scale expression tests on 50 mL cultures are necessary to optimize soluble protein overexpression.
3. Depending on the fusion protein chosen, the concentration of IPTG used for protein induction should be optimized in small-scale protein expression tests before the large-scale protein preparation.
4. Significant absorbance will depend greatly on the robustness of protein expression. When dealing with poorly expressed proteins, checking the ratio of the A_{280} and A_{260} of the peak eluted from the column proves helpful. Ratios tending toward 2.0 are excellent; however, ratios of greater than 1.4 are acceptable. Therefore, if a fraction contains an A_{280} of 0.2 and an A_{260} of 0.1, this would be a good fraction to collect. Conversely, an A_{280} of 0.2 and A_{260} of 0.2 would not. This is generally not an issue with proteins that are expressed very well, as their A_{280}s tend to be much greater (i.e., greater than 2.0).
5. To optimize the oligomerization of the protein nanorings, run the oligomerization reaction in several different ratios of the protein to dimerize. Additionally, the linker length will

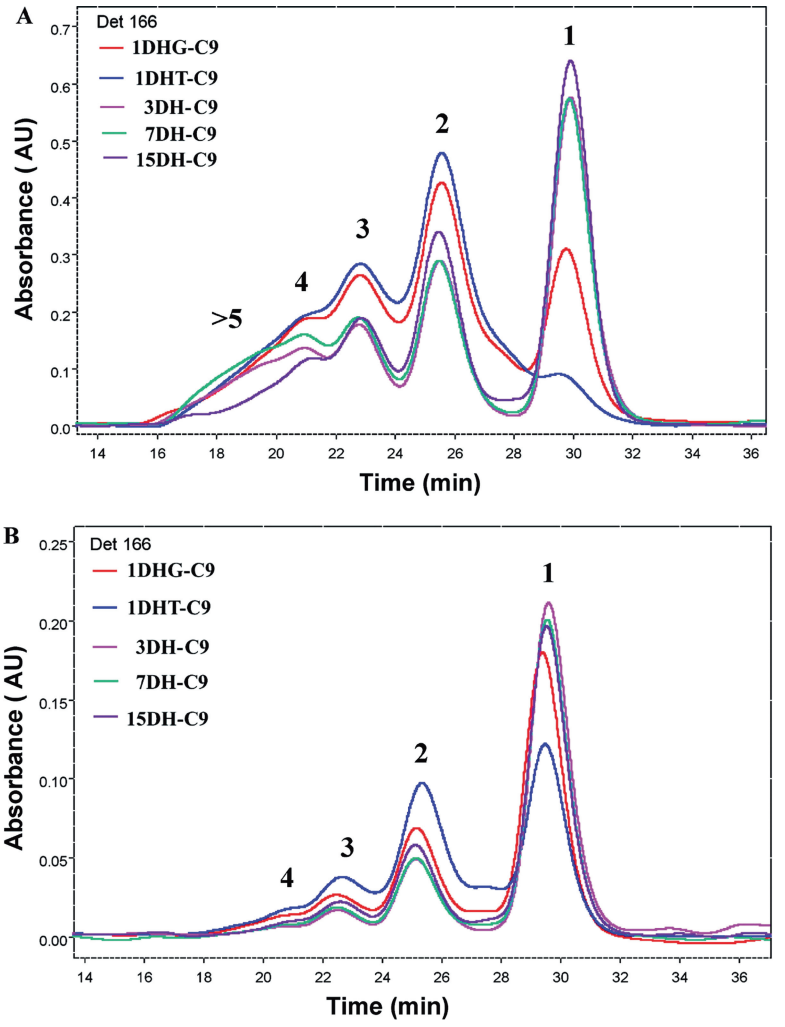

Fig. 2.5. Comparison of bis-MTX polymerized DHFR–hHint1 fusion proteins with different lengths of polypeptide linkers. Overlaid elution profiles for (**a**) 25 μM of protein mixed with 1 eq of bis-MTX and (**b**) 5 μM of protein mixed with 1 eq of bis-MTX. Figure reproduced from (5) with permission from the American Chemical Society. (To follow the absorbance shown above, lines are shown from top to bottom in this order for peak 1 position: 15DH-C9, 7DH-C9, 3DH-C9, 1DHG-C9 and 1DHT-C9).

affect the number of monomers present in the oligomeric ring (**Fig. 2.5**).

6. All the buffers for HPLC-SEC should be filtered through a 0.2 μm filter and degassed before usage. Make sure to equilibrate the column with P500 buffer until the UV baseline is stable before running protein samples. Lastly, after 10 gel filtration runs, wash the column at a flow rate of 0.5 mL/min with 25 mL 0.5 M NaOH, 25 mL deionized water, 25 mL 50% ethanol, 25 mL deionized water, and 50 mL P500 buffer.

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