Исследования по клеточной биологии

УДК 577.21

A. Mikhaylina, N. Lekontseva, A. Khairetdinova, N. Ilyina, V. Balobanov

A SIMPLE METHOD FOR OBTAINING A CHAPERONE WITH A VARIABLE NUMBER OF NON-NATIVE PROTEINS BINDING SITES[1]

Proteins are one of the key elements of the cell. Their correct folding and prevention of aggregation under stress conditions are ensured by chaperone proteins. Understanding the principles of functioning of chaperones makes it possible to create and modify them to solve research problems or use them in biotechnology. We created an artificial chaperone based on the apical domain of GroEL. In this work, we present an approach that allows you to vary the number of non-native proteins binding sites on this chaperone. This approach allowed us to estimate the stoichiometry and binding strength of the non-native protein by the chaperone we created. It was shown that reliable binding of non-native αLA requires its interaction with several apical domains of GroEL. At the same time, the dissociation constant of such a complex does not change significantly with an increase in the number of binding domains in the oligomer. Up to 4 αLA molecules can be attached to a complete heptameric ring of apical domains. In the future, the proposed tool can also be used not only to study chaperones, but also to obtain proteins with a combination of any functional domains.

Keywords: protein design; hetero-oligomer; multivalent binding; Sm-like proteins; chaperone.

DOI: 10.35634/2412-9518-2023-33-4-382-391

Binding of a non-native protein is the first stage of chaperone-assisted protein folding. This binding obeys the general laws of association/dissociation of protein complexes. The main feature of chaperone binding is that it is the result of a combination of many weak and nonspecific interactions [1]. This kind of interaction is called polyvalent. Polyvalent binding is also characteristic of other proteins. Multidomain proteins with multiple binding sites are widespread in nature. First, these are various antibodies. So IgA has four antigen binding sites, and IgM has ten. No less remarkable are multivalent hormone/receptor complexes [2; 3]. Protein multivalency can increase binding affinity, avidity and specificity compared to monovalent counterparts [4]. A multivalent interaction can be strong even if its individual bonds are weak [5; 6]. No less interesting is that multivalency significantly increases the sensitivity of the protein-ligand interaction to external conditions, which leads to a hypersensitive and highly nonlinear dependence of the binding strength on parameters such as temperature, pH or concentration of components [7; 8]. In biological systems, low-affinity proteins typically cluster together to achieve the required binding strength. From an evolutionary perspective, it seems convenient to combine several low-affinity single interactions to produce a collective stronger result. Additionally, tunable multivalent receptor-ligand interactions mediate differential responses to biological signals.

As can be seen from the above, the binding of several identical ligands by one protein and the binding of the same ligand by several binding sites in the literature are called the same term - multivalent binding. Therefore, before continuing the story, it is worth dwelling on the differences between these variants of multivalence. So: a typical IgG antibody binds two ligands at two independent binding sites and is therefore bivalent. The binding of a non-native protein to a chaperone, involving one ligand and several chaperone subunits, can also be called multivalent [9]. In an experiment, multivalent binding will be manifested by anomalous behavior of association/dissociation kinetic curves. In the case of independent binding of each ligand to each binding site, there will be no fundamental difference from the simple model (using the example of immunoglobulin) [10]. The only difference will be in the effective concentration and stoichiometry of the resulting complex. Significant differences will occur when one ligand binds to multiple binding sites (eg, a chaperone). The nonlinearity of the bond strength and the above-mentioned hyperselectivity will also appear here [11].

The theoretical description of the kinetics and thermodynamics of processes occur-ring during polyvalent interaction is complex. The theory is discussed in sufficient detail in the works [10; 12].

[1] This research was funded by RUSSIAN SCIENCE FOUNDATION, grant number RSF 22-24-00934.

Theoretical models can be used to obtain thermodynamic interaction parameters based on experimental data obtained using the different methods such as surface plasmon resonance [13], bio-layer Interferometry [14] or others[15; 16]. However, there is a rather serious problem - the more complex model leads more ambiguous in experimental data describing. The researcher needs to clearly understand the required limit of detail in describing the process. A simple comparison of several protein variants with each other is often required [17]. Determining the equilibrium dissociation constants of complexes also rarely requires great accuracy. At the same time, the excessive complexity of the used mathematical model can worsen the obtained result. In our work, we tried to simplify as much as possible the experimental methods used and the processing of results. The reader will be able to assess how far we succeeded in this.

Artificial chaperone ADGroEL_SacSm. The object of our research is the artificial chaperone ADGroEL_SacSm. The design principle of ADGroEL_SacSm was the attachment of the apical domain of the chaperone GroEL (ADGroEL) to the oligomeric scaffold of Sm-like protein from Sulfolobus acidocaldarius (SacSm). Analysis of the structure of SacSm shows that the simplest way to attach the target domain to it is to attach it with a flexible linker to the N- or C-terminus [18], which was done.

Sm-like proteins are present in the cells of bacteria, archaea and eukaryotic organisms [19; 20]. These are often RNA-binding proteins. They are also often attributed to the role of RNA chaperones. There are proteins with the number of monomers in the ring from 5 to 8. The structure of many of them is resistant to denaturants and increased temperature [21; 22]. They are an excellent basis for work like ours.

GroEL is a well-studied chaperone protein [23]. Structurally, it is a dodecamer of 2 rings of 7 subunits each. Each subunit consists of three domains: apical, intermediate and equatorial. ADGroEL plays a major role in non-native proteins binding. In addition, back in 1996 it was shown that monomeric ADGroEL, separated from the rest of the structure, retains chaperone function [24]. Structurally, ADGroEL is anchored at both ends of its polypeptide chain. Both ends are located in the equatorial region. At the same time, the interaction of ADGroEL with each other is quite weak, and they change mutual orientation when the equatorial domain bind ATP.

We combined SacSm and ADGroEL to construct the artificial chaperone ADGroEL_SacSm. The protein we obtained showed a good ability to bind non-native proteins and prevent their aggregation when heated. At the same time, monomeric ADGroEL did not exhibit such properties in our experiments [18].

The high stability of SacSm allows it to maintain its structure even at high urea concentrations [23]. We have also previously demonstrated efficient renaturation of ADGroEL attached to the SacSm protein scaffold. When renatured from the fully unfolded state (high concentration of guanidine hydrochloride), a significant difference in stability between the protein parts leads to a two-step folding. The first step is the folding of SacSm and the formation of a heptameric ring. The second step is folding of the ADGroEL attached to this ring. The possibility of such two-step folding determined the properties that we will use to assemble hetero-oligomers.

Task definition. As mentioned above, the heptomer ADGroEL_SacSm is capable of binding non-native proteins, but the monomeric ADGroEL is not. The question arises: how many ADGroELs collected together are enough to bind tightly? For full-size GroEL, the answer to this question was given in article [1]. As shown, the minimum element required for binding is two ADGroELs located next to each other. Does this work the same way in our artificial chaperone? In our protein, the mutual mobility of domains is higher than in GroEL; in addition, in the work mentioned above, binding was turned off by mutations, which does not exclude additional interactions. These questions led us to the idea of changing the number of binding domains in the oligomer. We developed and applied two simple methods. The first method is to obtain chaperones with different numbers of binding sites. The second allows one to evaluate the stoichiometry and strength of their binding to non-native proteins.

The significant difference in stability between the SacSm base and the attached AD-GroEL allows the following manipulations. When the fusion protein was fully un-folded in a solution with a high concentration of guanidine hydrochloride, SacSm (without ADGroEL attached) was added. Upon further folding, it was included in the ring of the oligomer base. This inclusion is random and proportional to the ratio of AD-GroEL_SacSm and empty SacSM. In such a situation, after complete renaturation, the output will be proteins with different amounts of ADGroEL in the ring. A remarkable feature of this system is its versatility and applicability to other attached domains, pro-vided they are capable of efficient renaturation.

To assess the stoichiometry of the resulting complexes and the strength of binding of their constituent proteins, we have developed a technique based on metal affinity chromatography and quantitative analysis of its results using SDS-gel electrophoresis.

Materials and Methods

Purification of the Fusion Protein ADGroEL_SacSm. The pET-22b vector carrying the ADGroEL_SacSm fusion protein gene was used for transformation in *E. coli* BL21(DE3)/pRARE to express the protein. *E. coli* strain BL21(DE3) cells were preliminarily co-transformed with the pRARE, which carried rare-codon tRNA genes (AUA, AGG, AGA, CUA, CCC, and GGA) to enhance the expression of fusion proteins, including an archaeal SacSm part that contained codons rarely used in *E. coli*.

The transformants were grown in an LB medium in the presence of ampicilline (100 μg/mL) and chloramphenicol (10 μg/mL), at 37 °C with an agitation of 180 rpm. Protein expression was induced at OD 600 nm = 0.6 – 0.8 o.u. with the addition of IPTG at a final concentration of 0.5 mM. The bacteria were harvested with centrifugation 3 h after in-duction.

Cell pellets were suspended in a solution containing 500 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM imidazole, 5 mM β-mercaptoethanol, and 6 M Guanidine-HCl. Cells were disrupted by sonication at 4 °C. Cell debris was removed by centrifugation at 15000× g for 30 min at 4 °C. Supernatant was loaded onto a Ni-NTA agarose (GE Healthcare, Uppsala, Sweden) column equilibrated with a solution containing 500 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 10 mM imidazole and 8 M Urea. ADGroEL_SacSmAP were eluted using a step gradient of imidazole (40 mM and 150 mM) in a solution containing 500 mM NaCl and 50 mM Tris-HCl (pH 8.0) and 6 M Urea. Fractions containing the protein were collected and purified using Q-Sepharose equilibrated with a solution containing 500 mM NaCl, 50 mM Tris-HCl (pH 8.0). Fractions containing the protein were concentrated and dialyzed against a solution containing 150 mM NaCl and 50 mM Tris-HCl (pH 8.0). The final purification step was size-exclusion chromatography on the Superdex 75 resin equilibrated with a solution containing 150 mM NaCl and 50 mM Tris-HCl (pH 8.0). Fractions containing the protein were concentrated and dialyzed against a solution containing 150 mM NaCl and 50 mM Tris-HCl (pH 8.0).

Expression and purification of SacSm. Competent BL21(DE3)/pLacIRARE *E. coli* were transformed with pProExHTb_SacSm and grown in LB media (100 mg/ml ampicillin and 10 mg/ml chloramphenicol) at 37 °C to an optical density at 600 nm (OD600) of ≈ 0.8. Then, overexpression of SacSm was induced by adding IPTG to a final concentration of 0.5 mM. After overnight incubation at 20 °C, cells were centrifuged at 14000× g for 30 min.

The cell pellet from the previous step was resuspended in Lysis Buffer (20 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 10 mM imidazole, 1 mM PMSF, 1 mM DTT, 0.1 % Triton X-100) and disrupted by sonication (Fisher Scientific, USA). The extract was cleared by centrifugation at 14000g for 30 min, then the supernatant was heated at 70 °C for 20 min and denatured *E. coli* proteins were removed by centrifugation (14000g, 40 min, 4 °C).

SacSm containing protein sample was loaded on a Ni-NTA Agarose (Qiagen) column equilibrated with 20 mM sodium phosphate buffer, pH 8.0, 0.2 M NaCl, 10 mM imidazole.

The His-tagged SacSm protein was eluted by applying a linear gradient of imidazole, from 10 mM to 250 mM. To proteolytically remove the His-tag SacSm-containing fractions were combined, concentrated and incubated with TEV protease at a 1:100 mass ratio of TEV:SacSmAP. To remove TEV protease from the sample, the solution was heated for 20 min at 65 °C, then cleared by centrifugation (14000g, 40 min, 4 °C) and reapplied to the Ni-NTA Agarose to remove cleaved His-tags. The flow-through of SacSmAP was concentrated and applied to Superdex 75, equilibrated with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM DTT.

Obtaining hetero-oligomers by the denaturation/renaturation. The schematic diagram of heterooligomers obtaining is shown in Figure 1. ADGroEL_SacSm and SacSm proteins were mixed in different molar ratios. Dry Guanidine hydrochloride was added to the protein solution up to saturation. Then a solution of 20 mM Hepes-NaOH pH 7.5, NaCl 200 mM was gradually added to the protein solution with 30 minutes incubation at each step until Guanidine hydrochloride concentration achieved 1 M. The resulting mixture was applied to a chromatography column with Ni-Focurose metal chelate resin (Elabscience, China). The column was washed with buffer contained 20 mM Hepes-NaOH pH 7.5, NaCl 200 mM. The target protein was eluted from the Ni-Focurose column with the same solution with the addition of 500 mM imidazole.

The separation of hybrid forms of proteins was carried out with size-exclusion chromatography using Superdex 200 Increase 10/300 GL column. Chromatography was performed on the AKTA Basic FPLC system (Amersham, Sweden). The volume of protein injected was 300 μl, with a total protein concentration of 3 – 4 mg/ml. Working solution contained 20 mM Hepes-NaOH pH 7.5, NaCl 200 mM. The flow rate was 0.4 ml/min. Proteins containing varying amounts of ADGroEL were separated according to their molecular weight. The volume of each fraction was 200 μl.

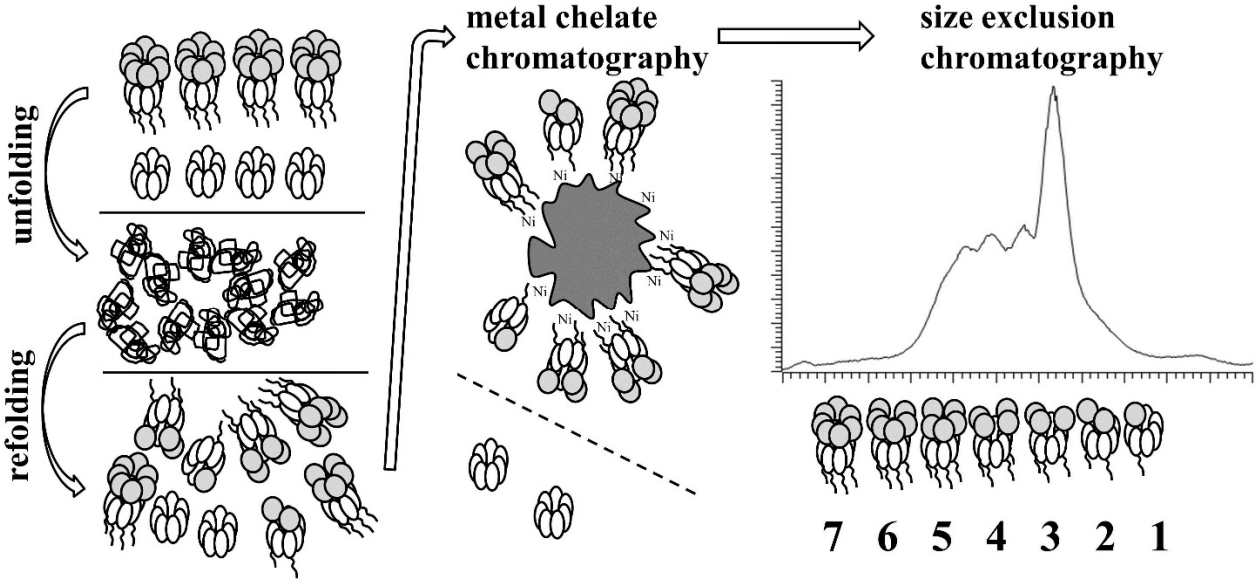

Fig. 1. A scheme for obtaining heterooligomers with a different number of binding domains.
A protein with a binding domain has his-tag and protein without such domain does not have it.
After collaborative refolding, proteins having a binding domain are purified on a metallochelate resin and separated by size exclusion chromatography

Non-native protein binding study. The schematic diagram of non-native protein binding experiment is shown in Figure 2. We used denatured alpha-Lactalbumin (αLA) (Sigma, USA) as a model substrate for protein binding assay. αLA was in solution contained 20 mM Hepes-NaOH pH 7.5, NaCl 200 mM, Urea 6 M, beta-mercaptoethanol 10 mM before use. αLA was added to a solution of ADGroEL_SacSm/SacSm hetero-oligomers in a ratio of 7 αLA molecules per one ring of ADGroEL_SacSm/SacSm in a buffer solution contained 20 mM Hepes-NaOH pH 7.5, NaCl 200 mM, beta-mercaptoethanol. In the presence of a reducing agent, αLA is unable to assume its native structure. The protein solution was incubated for 10 minutes followed by applying to Ni-Focurose and unbound αLA was washed off with the same buffer solution. Proteins bound to the Ni-Focurose were eluate with a buffer solution 20 mM Hepes-NaOH pH 7.5, NaCl 200 mM, Imidazole 500 mM and analyzed by SDS-PAGE.

SDS-PAGE was used to analyze the purity and quantitative characterization of the hybride ADGroEL_SacSm/SacSm proteins.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method with some modifications.

The separating gel contained: 15 % acrylamide (AA:MBA ratio is 39:1), 0.375 M Tris-HCl, pH 8.8, 0.1 % SDS. 0.1 % TEMED and 0.1 % APS were added for polymerization.

The stacking gel contained: 6 % acrylamide (AA:MBA ratio is 39:1), 0.125 mM Tris-HCl, pH 6.8, 0.1 % SDS. 0.1 % TEMED and 0.1 % APS were added for polymerization.

The gel was carried into the gel plates measuring 7 × 8 cm and 0.75 mm thick.

Samples were prepared by adding Laemmli sample buffer in an amount of 1/5 of the sample volume and boiled for 5 min. Laemmli sample buffer contained 0.3 M Tris-HCl, pH 6.8, 10 % SDS, 25 % β-mercaptoethanol, 30 % glycerol, 0.1 % BromPhenol Blue.

The running buffer used was Tris/Glycine/SDS and contained 25 mM Tris, 192 mM Glycine, 0.1 % SDS. Gels were run in the “Mini Protean II” electrophoresis cell from Bio-Rad (USA) at 200 V until the dye front reached the bottom of the gel.

After electrophoresis, gels were stained in 0.05 % Coomassie G-250 solution in 30 % Ethanol and 10 % Acetic Acid. The background color was washed off by boiling in 5 % Acetic Acid.

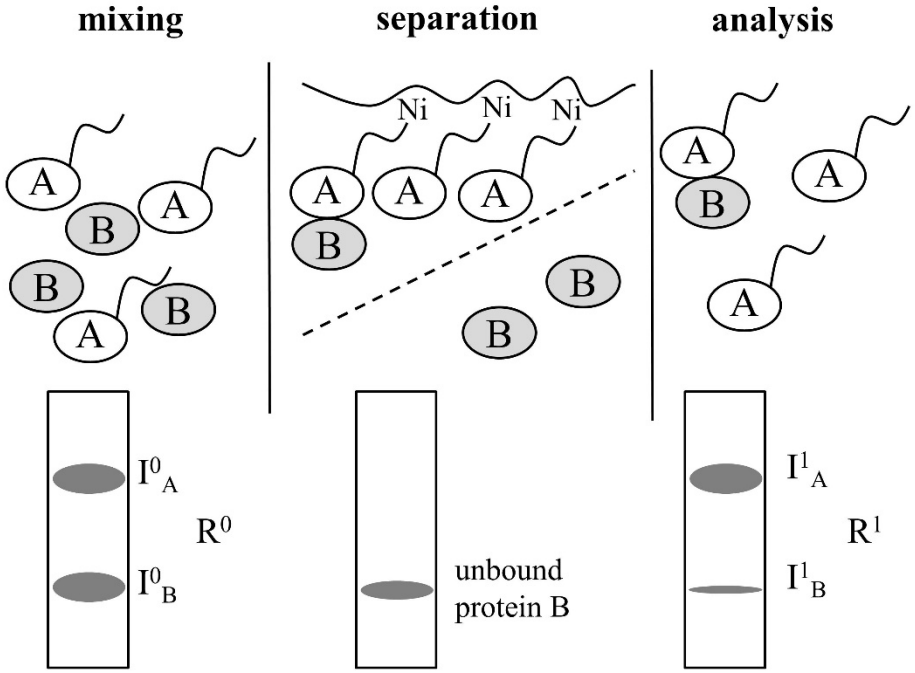

Fig. 2. The study design for investigating protein-protein binding. Protein A has his-tag and protein B does not. After co-incubation, protein B not bounded to A is separated by metallochelate chromatography. Each stage was analyzed by SDS-PAGE.

SDS-PAGE images analysis and calculation of equilibrium dissociation constants. To quantitatively analyze the ratios of ADGroEL_SacSm and SacSm in hetero-oligomers and analyze the binding of αLA, the color intensities of the corresponding bands on the SDS-PAGE images were determined. The determination was carried out using the TotalLab Software (nonlinear dynamics, UK). The color intensity of the corresponding bands (I_A and I_B)was calculated after subtracting the background color intensity. After this, considering the color intensity to be proportional to the protein mass in the band and taking into account its molecular weight (Mr_A and Mr_B), we can introduce the following relations (where N_A and N_B – number of molecules that is proportional to molar concentration [A] and [B]) :

$$R^0 = \frac{N_A^0}{N_B^0} = \frac{I_A^0/Mr_A}{I_B^0/Mr_B} = \frac{[A]^0}{[B]^0}; \qquad R^1 = \frac{N_A^1}{N_B^1} = \frac{I_A^1/Mr_A}{I_B^1/Mr_B} = \frac{[A]^1}{[B]^1};$$

Since protein A fully binds to the resin and protein B binds only as part of the complex, then:

$$\frac{[A]^1}{[B]^1} = \frac{[A]^0}{[AB]}$$

For the simplest variant of determining the dissociation constant, we will express the necessary concentration values through the relations that we have introduced

$$K_d = \frac{[A][B]}{[AB]}$$

$$[AB] = \frac{[A]^0}{R^1}$$

$$[A] = [A]^0 - [AB] = [A]^0 - \frac{[A]^0}{R^1} = [A]^0\left(1 - \frac{1}{R^1}\right)$$

$$[B] = [B]^0 - [AB] = [B]^0 - \frac{[A]^0}{R^1} = [A]^0\left(\frac{[B]^0}{[A]^0} - \frac{1}{R^1}\right) = [A]^0(\frac{1}{R^0} - \frac{1}{R^1})$$

$$K_d = \frac{[A]^0\left(1 - \frac{1}{R^1}\right)[A]^0(\frac{1}{R^0} - \frac{1}{R^1})}{[A]^0/R^1} = R^1[A]^0(1 - \frac{1}{R^1})(\frac{1}{R^0} - \frac{1}{R^1})$$

Thus, knowing the total concentration of protein A and determining the ratio of components before and after the separation of unbound protein B, we can estimate the dissociation constant of the resulting complex.

Results and Discussion

Preparation of hetero-oligomers. Hetero-oligomers were obtained according to the method described in the Materials and Methods section. Different ratios of ADGroEL_SacSm and SacSm were tested. A change in this ratio expectedly leads to a shift in the distribution of proteins towards more or less ADGroEL_SacSm in the hetero-oligomer. With a starting ADGroEL_SacSm / SacSm ratio of 1/3, the distribution allowed us to obtain almost all variants in an acceptable quantity. We separated the mixture of the resulting hetero-oligomers into fractions by size-exclusion chromatography (fig. 3 A). Separation is more efficient for hetero-oligomers with small amount of ADGroEL domains, despite the same molecular weight step (per molecular weight of one ADGroEL), this is due to a smaller percentage change in the hydrodynamic size with a larger number of domains. Electropherogram patterns of the obtained fractions analysis made it possible to calculate the ADGroEL_SacSm / SacSm ratios in the obtained hetero-oligomers (fig. 3 B and table 1). In general, the method of obtaining and analyzing is quite simple and intuitive. Its disadvantages include the inability to control the relative position of the ADGroEL on the ring. When transferring the technique to other proteins, their renaturation ability will also be a limitation.

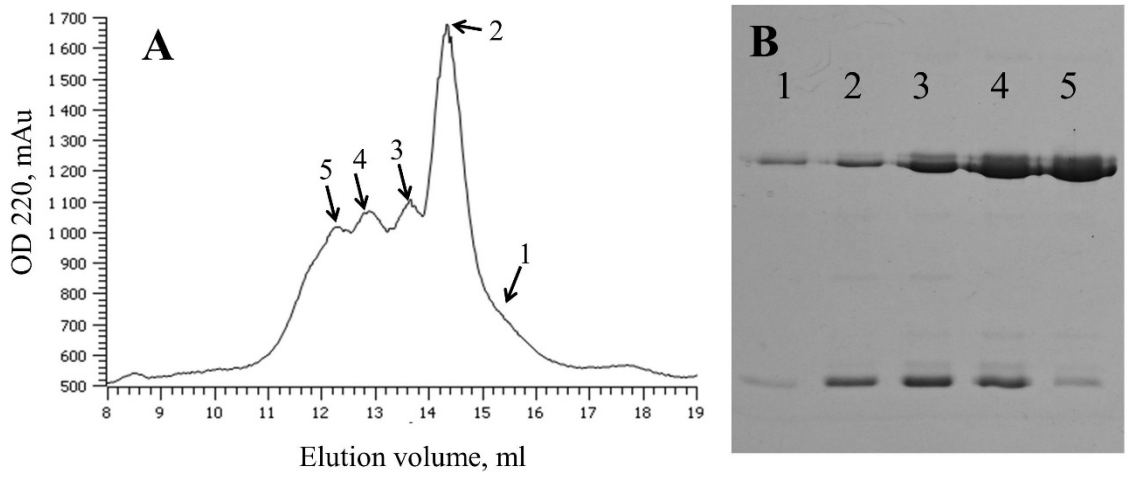

Fig. 3. Results of separation of ADGroEL_SacSm / SacSm heterooligomers with different numbers of binding domains by size-exclusion chromatography. Chromatogram – A and SDS-PAGE analysis of the corresponding fractions – B.

The quantitative assessment is given in table 1.

Table 1

Calculation of the ADGroEL_SacSm / SacSm ratios in the obtained hetero-oligomers

Fraction number (see fig. 3)	Color intensity of ADGroEL_SacSm bands	Color intensity of SacSm bands	ADGroEL_SacSm / SacSm molar ratio	ADGroEL_SacSm number per ring
1	418449	–	–	–
2	600442	602505	0,28	1.6
3	1818168	879939	0,59	2.6
4	2942202	806784	1,04	3.6
5	3202075	197672	4,61	5.8

Non-native protein binding assay. We used αLA as a model of non-native protein. In the presence of a reducing agent, αLA is unable to assume its native structure.We obtained complexes of chaperones and non-native proteins by their joint incubation. To evaluate the binding of non-native proteins, we separate bound and unbound non-native proteins on a Ni-Focurose metal chelate resin. αLA does not bind to this resin. ADGroEL_SacSm and obtained hetero-oligomers have a His-tag and therefore can binding to resin.

After washing the not bound proteins, only αLA bound to ADGroEL_SacSm remains bound to Ni-Focurose. The results show that hetero-oligomers containing only 2 ADGroEL_SacSm subunits do not bind to non-native αLA (fig. 4). This experiment is also a control for the binding of αLA to the Ni-Focurose and to the SacSm oligomer, which serves as the scaffold of the hetero-oligomer. In our case, such binding does not occur. αLA reliably binds to hetero-oligomers with three or more ADGroEL_SacSm in their composition (fig. 4 and table 2).

To solve the problem of assessing the stoichiometry of the resulting complex and the binding strength of the substrate protein, we used the same experimental approach. Of course, more precise methods could have been used, such as SPR or MST. But a rough estimate was enough for us. In addition, we wanted to obtain a simpler and cheaper experimental technique. And we got it.

The results obtained show that binding is detected only for a hybrid containing at least 3 ADGroEL in the ring. In this case, the binding stoichiometry is close to the ratio of one αLA per two ADGroEL. This can be explained by the binding of two adjacent ADGroELs of one αLA. Which correlates well with the data for a full-size GroEL from the article [1]. However, discrepancies with the literature data were also found. Thus, the stoichiometry of the interaction of full-length GroEL with non-native proteins is estimated as one non-native protein per one GroEL ring, that is, 7 ADGroELs. Our artificial chaperon binds 4 αLA molecules per complete ring. Most likely, this can be explained by the greater mobility of the ADGroELs relative to each other, which reduces the competition between the bound molecules.

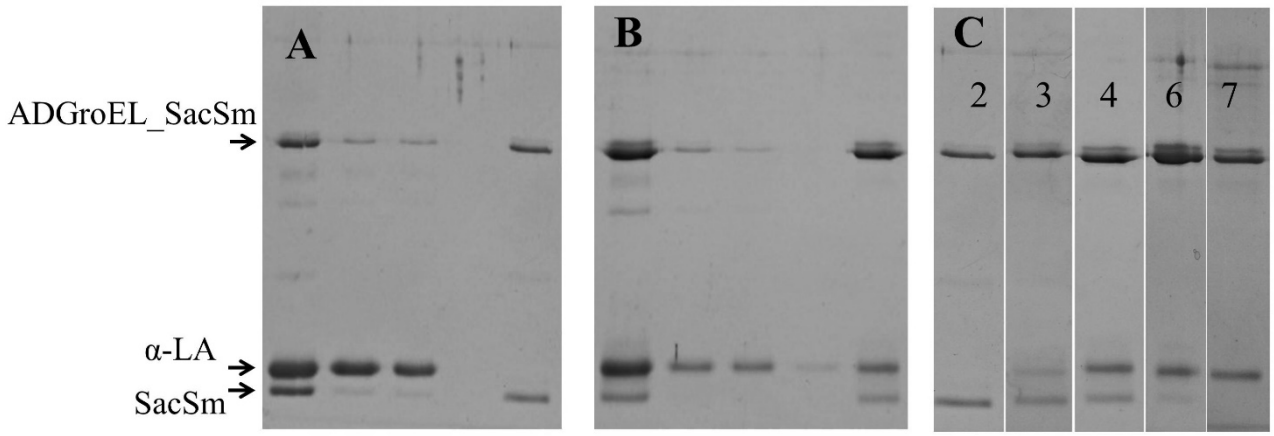

Fig. 4. Analysis of the binding of non-native proteins by chaperone. The first line in fields A and B is the initial mixture, the last line is after the separation of the unbound protein. Intermediate lines – resin washing. Field C is a combination of lines after separation of an unbound protein. The number of binding domains in heterooligomer indicated on the corresponding line.

Table 2

Calculation of stoichiometry and dissociation constants based on the data in fig.4

ADGroEL_SacSm number per ring	ADGroEL_SacSm /αLA molar ratio, initial	ADGroEL_SacSm /αLA molar ratio, in complex	αLA molecules per hetero-oligomer ring	Kd (M)
2	0,32			
3	0,50	2,49	0,96	$4*10^{-6}$
4	0,72	2,14	1,75	$3*10^{-6}$
6	0,90	2,34	2,44	$4*10^{-6}$
7	0,57	1,57	4,41	$3*10^{-6}$

We estimated the dissociation constant using the described above method. We calculated molar ratios of proteins before and after separation on the Ni-Focurose using color intensities of electropherogram bands. This approach is based on the assumption that the color intensity is proportional to the mass of the protein in this band. I agree, the assumption is quite rough. However, it is often necessary to estimate the dissociation constant from the order value and to compare the efficiency of interaction between different variants of molecules. Such accuracy is quite achievable for the applied method. And it is quite sufficient for comparing dissociation constants determined uniformly among themselves by the same technique.

The calculation results are shown in the table. In the calculation, we consider the concentration of ADGroEL_SacSm as the concentration of binding sites. The result shows that the dissociation constant practically does not change when the proportion of ADGroEL_SacSm in the hetero-oligomer changes.

The totality of the data obtained suggests the following picture: the interaction of a single ADGroEL with non-native proteins is quite weak. This prevents it from being detected by our methods. But such interaction is sufficient for it to exhibit chaperone activity, as follows from the literature data [24]. When several ADGroELs are located closely on a single base, non-native proteins can interact with several domains simultaneously and, accordingly, the stability of such a complex increase exponentially. Our model protein αLA is small and cannot interact with more than two ADGroELs, which is manifested by the absence of a change in the dissociation constant with an increase in the proportion of ADGroEL_SacSm in the hetero-oligomer. At the same time, its small size allows up to 4 molecules to be placed on the complete heptameric ADGroEL_SacSm ring.

Conclusions

Thus, the method of obtaining a multivalent protein with a variable number of binding sites showed good results and ease of use. A simple method for assessing binding strength has also demonstrated its utility. Comparison of these methods with alternative approaches shows their advantages.

Varying the number of binding sites on a protein by assembling hetero-oligomers can significantly reduce the cost and time spent compared to alternative approaches. You can agree, obtaining an individual genetic construct for each variant and isolating each variant of the corresponding proteins is much more difficult. In addition, this approach is also applicable to obtain complexes with a combination of different functional domains. In vitro production is possible as described in our article through denaturation and joint stepwise renaturation. In vivo production is possible through coexpression of components and self-assembly in the cell.

The proposed method for estimating the stoichiometry of the resulting complexes and the strength of interaction in them is rather coarse. But if you compare our approach with alternative methods, it becomes clear that its accuracy is sufficient for many tasks. At the same time, it does not require expensive equipment and qualified staff. If greater accuracy is required, then our proposed approach may well be used as the first stage of searching for conditions for further experiments by other methods.

REFERENCES

1. Farr, G.W., Furtak, K., Rowland, M.B., Ranson, N.A., Saibil, H.R., Kirchhausen, T., Horwich, A.L. Multivalent Binding of Nonnative Substrate Proteins by the Chaperonin GroEL, in *Cell*, 2000, vol. 100, pp. 561-573.
2. Merminod, S., Edisonb, J.R., Fanga, H., Hagana, M.F., Rogers, W.B. Avidity and surface mobility in multivalent ligand–receptor binding, in *Nanoscale*, 2021, vol. 13, no. 29, pp. 12602-12612.
3. Xia, X., Zhang, G., Ciamarra, M.P., Jiao, Y., Ni, R. The Role of Receptor Uniformity in Multivalent Binding, in *JACS Au*, 2023, vol. 3, pp. 1385-1391.
4. Curk, T., Dobnikar, J., Frenkel, D. Design Principles for Super Selectivity using Multivalent Interactions, in *Multivalency: Concepts, Research & Applications*, 2018, vol. 3, pp. 75-101.
5. Martos, V., Castreno, P., Valero, J., Mendoza, J. Binding to protein surfaces by supramolecular multivalent scaffolds, in *Current Opinion in Chemical Biology*, 2008, vol. 12, pp. 698-706.
6. Scheepers, M.R.W., IJzendoorn, L.J., Prins, M.W.J. Multivalent weak interactions enhance selectivity of interparticle binding, in *PNAS*, 2020, vol. 117, no. 37, pp. 22690-22697.
7. Csizmar, C.M., Petersburg, J., Perry, T.J., Rozumalski, L., Hackel, B.J., Wagner, C.R. Multivalent Ligand Binding to Cell Membrane Antigens: Defining the Interplay of Affinity, Valency, and Expression Density, in *J. Am. Chem. Soc.,* 2018, vol. 1, pp 1-36.

8. Dernedde, J. Multivalency in Biosystems, in *Multivalency: Concepts, Research & Applications*, 2018, vol. 4, pp. 103-120.
9. Marchenkov, V., Gorokhovatsky, A., Marchenko, N., Ivashina, T., Semisotnov, G. Back to GroEL-Assisted Protein Folding: GroES Binding-Induced Displacement of Denatured Proteins from GroEL to Bulk Solution, in *Biomolecules*, 2020, vol. 10, no. 1, pp. 162-174.
10. Errington, W.J., Bruncsics, D., Sarkar, C.A. Mechanisms of noncanonical binding dynamics in multivalent protein–protein interactions, in *PNAS*, 2019, vol. *116, no. 51*, pp. 25659-25667.
11. Tan, Z.C., Meyera, A.S. A general model of multivalent binding with ligands of heterotypic subunits and multiple surface receptors, in *Math Biosci*., 2021, vol. 342, pp. 108714.
12. Bruncsics, B., Errington, W.J., Sarkar, C.A. MVsim is a toolset for quantifying and designing multivalent interactions, in *Nature Communications*, 2022, vol. 13, no. 5029, pp. 1-13.
13. Douzi, B. Protein-Protein Interactions: Surface Plasmon Resonance, in *Methods. Mol. Biol*., 2017, vol. 1615, 257-275.
14. Sultana, A., Lee, J.E. Measuring protein-protein and protein-nucleic Acid interactions by biolayer interferometry, in *Curr. Protoc. Protein. Sci.,* 2015, vol. 19, no. 25, pp. 1-26.
15. Jerabek-Willemsen, M., André, T., Wanner, R., Roth, H.M., Duhr, S., Baaske, P., Breitsprecher, D. MicroScale Thermophoresis: Interaction analysis and beyond, in *Journal of Molecular Structure*, 2014, vol. 1077, pp. 101-113.
16. Pierce, M.M., Raman, C.S., Nall B.T. Isothermal titration calorimetry of protein-protein interactions, in *Methods*, 1999, vol. 19, no. 2, pp. 213-221.
17. Linke, P., Amaning, K., Maschberger, M., Vallee, F., Steier, V., Baaske, P., Duhr, S., Breitsprecher, D., Rak, A. An Automated Microscale Thermophoresis Screening Approach for Fragment-Based Lead Discovery, in *J Biomol Screen*, 2016, vol. 21, no. 4, pp. 414-21.
18. Mikhaylina A.O., Lekontseva N.V., Marchenkov V.V., Kolesnikova V.V., Khairetdinova A.R., Nikonov O.S., Balobanov V.A. The New Functional Hybrid Chaperone Protein ADGroEL-SacSm, in *Molecules*, 2023, vol. 28, pp. 6196.
19. Lekontseva, N.V., Stolboushkina, E.A., Nikulin, A.D. Diversity of LSM Family Proteins: Similarities and Differences, in *Biochemistry (Moscow)*, 2021, vol. 86, pp. S38-S49.
20. Mura, C., Randolph, P.S., Patterson, J., Cozen, A.E. Archaeal and eukaryotic homologs of Hfq, in *RNA Biology*, 2013, vol. 10, no. 4, pp. 636-651.
21. Murina, V.N., Melnik, B.S., Filimonov, V.V., Ühlein, M., Weiss, M.S., Müller, U., Nikulin, A.D. Effect of Conserved Intersubunit Amino Acid Substitutions on Hfq Protein Structure and Stability, in *Biochemistry (Moscow)*, 2014, vol. 79, no. 5, pp. 469-477.
22. Horovitz, A., Reingewertz, Y.H., Cuéllar, J., Valpuesta, J.M. Chaperonin Mechanisms: Multiple and (Mis)Understood? in *Annu. Rev. Biophys*., 2022, vol. 51, pp. 115–33.
23. Marchenkov, V., Lekontseva, N., Marchenko, N., Kashparov, I., Murina, V., Nikulin, A., Filimonov, V., Semisotnov, G. The Denaturant- and Mutation-Induced Disassembly of Pseudomonas aeruginosa Hexameric Hfq Y55W Mutant, in *Molecules,* 2022, vol. 27, no. 12, pp. 3821.
24. Zahn, R., Buckle, A.M., Perrett, S., Johnson, C.M, Corrales, F.J., Golbik, R., Fersht, A.R. Chaperone activity and structure of monomeric polypeptide binding domains of GroEL, in *Proc. Natl. Acad. Sci. USA,* 1996, vol. 93, no. 26, pp. 15024-15029.

Received 04.12.2023

Mikhaylina A.O., Candidate of Biology, Researcher
E-mail: alisamikhaylina@vega.protres.ru

Lekontseva N.V., Candidate of Biology, Researcher
E-mail: lekontseva@vega.protres.ru

Khairetdinova A.R., Senior laboratory assistant
E-mail: khairetdinova.studies@gmail.com

Ilyina N.B., research engineer
E-mail: nelly.ilyina@mail.ru

Balobanov V.B., Candidate of Science in Physics and Mathematics, Researcher
E-mail: balobanov@phys.protres.ru

Institute of Protein Research, Russian Academy of Sciences
Institutskaya st., 4, Pushchino, Russia, 142290

А.О. Михайлина, Н.В. Леконцева, А.Р. Хайретдинова, Н.Б. Ильина, В.А. Балобанов

ПРОСТОЙ МЕТОД ПОЛУЧЕНИЯ ШАПЕРОНА С ПЕРЕМЕННЫМ ЧИСЛОМ САЙТОВ СВЯЗЫВАНИЯ НЕНАТИВНЫХ БЕЛКОВ

DOI: 10.35634/2412-9518-2023-33-4-382-391

Белки являются одним из ключевых элементов клетки. Их правильное сворачивание и предотвращение агрегации в условиях стресса обеспечивают белки-шапероны. Понимание принципов функционирования шаперонов позволяет модифицировать и создавать новые шапероны для решения исследовательских задач и применения в биотехнологии. Мы создали искусственный шаперон на основе апикального домена GroEL. В данной работе мы представляем подход, позволяющий изменять количество сайтов связывания ненативных белков на этом шапероне. Такой подход позволил нам оценить стехиометрию и силу связывания ненативного белка созданным нами шапероном. Показано, что для прочного связывания ненативной αLA необходимо его взаимодействие с несколькими апикальными доменами GroEL. В то же время константа диссоциации такого комплекса существенно не меняется при увеличении числа связывающих доменов в олигомере. С полным гептамерным кольцом апикальных доменов может быть связано до 4 молекул αLA. В будущем предложенный в данной работе метод также можно будет использовать не только для изучения шаперонов, но и для получения белков с комбинацией любых функциональных доменов.

Ключевые слова: белковый дизайн, гетеро-олигомер, мультивалентное связывание, Sm-подобные белки, шаперон.

Поступила в редакцию 04.12.2023

Михайлина Алиса Олеговна, кандидат биологических наук, научный сотрудник
E-mail: alisamikhaylina@vega.protres.ru

Леконцева Наталья Владимировна, кандидат биологических наук, научный сотрудник
E-mail: lekontseva@vega.protres.ru

Хайретдинова Альбина Равильевна, старший лаборант
E-mail: khairetdinova.studies@gmail.com

Ильина Нелли Борисовна, инженер-исследователь
E-mail: nelly.ilyina@mail.ru

Балобанов Виталий Александрович, кандидат физико-математических наук, научный сотрудник
E-mail: balobanov@phys.protres.ru

Федеральное государственное бюджетное учреждение науки Институт белка Российской академии наук
142290, Россия, г. Пущино, ул. Институтская, 4