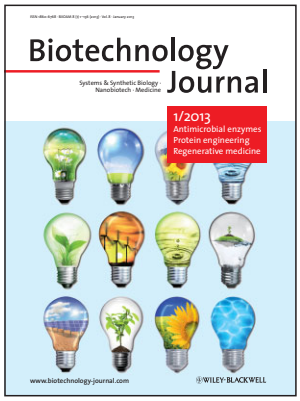

Cover illustration

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Review

Protein refolding using chemical refolding additives

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In laboratories and manufacturing settings, a rapid and inexpensive method for the preparation of a target protein is crucial for promoting resesrach in protein science and engineering. Inclusion-body-based protein production is a promising method because high yields are achieved in the upstream process, although the refolding of solubilized, unfolded proteins in downstream processes often leads to significantly lower yields. The most challenging problem is that the effective condition for refolding is protein dependent and is therefore difficult to select in a rational manner. Accordingly, considerable time and expense using trial-and-error approaches are often needed to increase the final protein yield. Furthermore, for certain target proteins, finding suitable conditions to achieve an adequate yield cannot be obtained by existing methods. Therefore, to convert such a troublesome refolding process into a routine one, a wide array of methods based on novel technologies and materials have been developed. These methods select refolding conditions where productive refolding dominates over unproductive aggregation in competitive refolding reactions. This review focuses on synthetic refolding additives and describes the concepts underlying the development of reported chemical additives or chemical-additive-based methods that contribute to the emergence of a universal refolding method.

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1 Introduction

With the success of the human genome project, many researchers are interested in the functional analysis and application of the gene products: proteins. In this context, the ability to rapidly and inexpensively convert gene sequences into their corresponding proteins is a significant advantage for both protein science and protein engineering [1]. Hence, processes in protein production are now increasingly more important both in the laboratory and in industrial settings. In upstream processes, there are numerous protein expression systems using various host cells, such as bacteria, yeast, insect cells, mammalian cells, and cell-free systems, to produce the target protein. Among them, *Escherichia coli* remains the first choice for host cell expression of proteins that do not require post-translational modifications. This is because of (i) low cultivation costs; (ii) rapid growth; and (iii) high expression levels of the target protein [2, 3]. Additionally, in the *E. coli* system, recombinant proteins can be produced as inclusion bodies, which are biologically inactive aggregates of high-purity recombinant protein [4]. The formation of inclusion bodies simplifies the recovery steps in downstream processes through solid–liquid separation and enables the expression of toxic proteins by inactivating

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Abbreviations: ACA, artificial chaperone-assisted; **L-ArgHCl**, L-arginine hydrochloride; **GdnHCl**, guanidine hydrochloride; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **PDI**, protein disulfide isomerase

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them in the host cells [2]. The former benefit is an extremely attractive feature for commercial-scale production. Moreover, many products on the market are produced as inclusion bodies in the cytoplasm of *E. coli* [3].

Conversely, inclusion-body-based systems also include a severe bottleneck, which is the requirement of a protein renaturation step from inclusion bodies [1–3]. To obtain biologically active protein, inclusion bodies are first solubilized by chemical denaturants. Strong chaotropes, such as urea and guanidinium hydrochloride, or strong detergents are employed to weaken the noncovalent interactions among proteins. In the case of target proteins with cysteine residues, strong reductants are used to break intermolecular disulfide bonds (S–S bonds). Such a solubilization step is now relatively easy to perform because well-established methods are applicable to any protein. However, under these solubilization conditions, the intramolecular interactions required for the formation of native protein structures rupture and solubilized proteins are often obtained in a flexible, random-coil state [5]. Therefore, the solubilized polypeptide chains have to be refolded into their correct structures to recover their native activities. The efficient conditions required for refolding generally have to meet the following requirements:

(i) the concentration of the denaturants should be reduced to a level where intramolecular noncovalent interactions, such as hydrogen bonding, hydrophobic interactions, and salt bridges, are retained;

(ii) the refolding process should be performed under an appropriate oxidizing environment to form the correct S–S bonds; and

(iii) the concentration of the denatured protein in the refolding buffer should be maintained at a low level to avoid intermolecular aggregation. To meet these requirements, a laborious and time-consuming trial-and-error approach for optimization of the refolding conditions is often required. In particular, the third requirement is critical to increase the refolding yield because protein refolding is a kinetically competitive process between productive folding and unproductive aggregation. Therefore, numerous technologies were developed to avoid aggregation in the refolding process [1–5].

The simple dilution of a solubilized protein solution has been the most frequently used method for protein refolding. In this method, a solubilized, denatured protein solution is diluted by a hundred- to a thousand-fold with an appropriate buffer. Reduction in the concentrations of both the denaturant and protein is simultaneously achieved by an extremely simple protocol. Protein folding is a first-order reaction with respect to protein concentration, whereas aggregation is a second- or higher-order reaction [6, 7]. This is because higher concentrations of protein enhance the probability of the collision of proteins, resulting in higher aggregation rates. Accordingly, by adequately decreasing the protein concentration (typically below 100 μg/mL), high-yield refolding with the suppression of protein aggregation was achieved [1, 7]. In addition, when a proper oxidative refolding buffer is employed in the refolding process, the three fundamental requirements for efficient refolding are simply accomplished by high protein dilution. However, considering the cost, time, and loss of protein in the following condensation process, protein refolding at higher concentrations is desired on both the laboratory and industrial scale. Moreover, in industry, high dilution has a cost disadvantage derived from huge reactors, large volumes of refolding buffer, and disposal of huge volumes of waste required [3, 8]. Most importantly, it is often the case that simple dilution cannot adequately suppress protein aggregation and leads to extremely low protein yields, even at the minimal concentration required to proceed to the next experimental or production step.

Such difficult-to-refold proteins have been reported to form agglutinative refolding intermediates in the folding process [6, 9, 10]. These intermediates are partially folded with secondary structures and their tertiary packing through the interaction of hydrophobic patches is molten [11]. Based on this knowledge, the competitive model between refolding and aggregation in a refolding process has been proposed as follows (Fig. 1): Initially, partially folded intermediates are quickly formed through intramolecular hydrogen bonds by decreasing the denaturant concentration. This is followed by the simultaneous refolding and aggregation of these intermediates, mainly through intra- and intermolecular hydrophobic interactions, respectively. In this kinetically competitive reaction, aggregation dominates over refolding when the refolding rate is slower. Here, the rate of refolding involving disulfide exchange reactions or proline isomerization is extremely slow [12–14]. Therefore, in the simple dilution refolding of proteins with more than two disulfide bonds, aggregation often dominates and leads to low refolding yields (Fig. 1). Most secreted proteins, such as interleukins and growth factors, contain a number of disulfide bonds in the native state to stabilize their tertiary structures [15]. Accordingly, such secreted proteins, which are important in the medical and pharmaceutical fields, are difficult targets for refolding due to their slow refolding rates.

To perform high-yield and high-concentration refolding, various refolding methods and protocols have been developed, as described in many excellent reviews [1–5]. Previous developments can be divided broadly into the following three categories: (i) development of methods to remove denaturants from the solubilized protein solution; (ii) methods involving the management of the physical conditions used during the refolding process; and (iii) methods in which refolding additives are present for suitable solution conditions of refolding processes. Herein, progress in the first two categories are only briefly introduced because an excellent review clearly introducing such progress was recently published [3]. We focus on the final category, especially on the idea of developing new re-

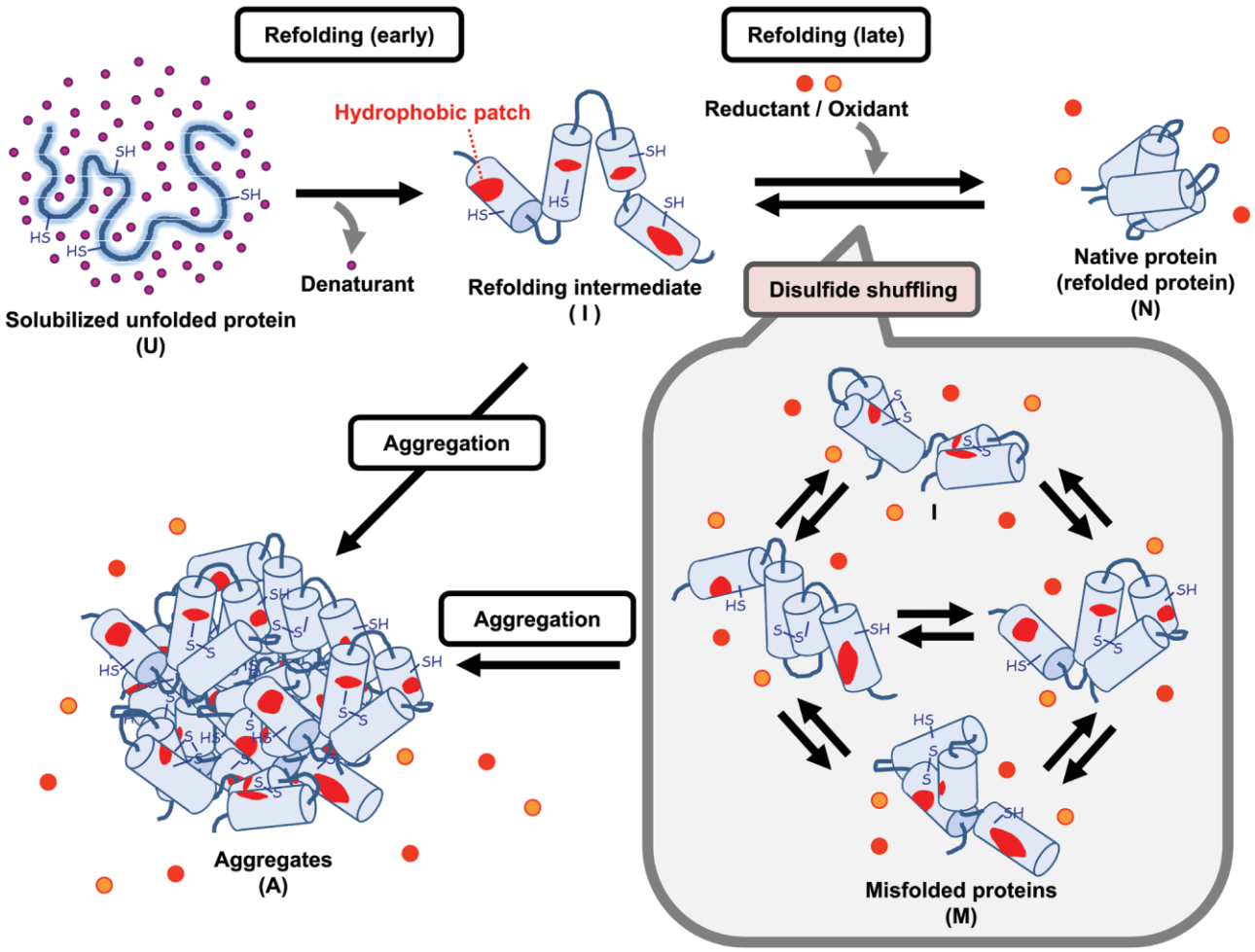

Figure 1. Schematic illustration of oxidative refolding of a difficult-to-refold protein. Partially folded intermediates are quickly formed by removal of denaturants, and subsequent refolding and aggregation of these intermediates competitively occurs mainly through the interactions of hydrophobic patches on the surfaces of proteins. The later step of oxidative refolding includes disulfide bond shuffling, which is catalyzed by reductants and oxidants, and such a "slow" reaction enhances aggregation in the competitive reaction between aggregation and refolding.

folding additives or additive-induced conditions oriented for practical use, rather than for scientific interest. By sharing such ideas with researchers who are developing new methodologies for protein refolding, we hope this review will contribute to the creation of breakthrough technologies for efficient protein refolding. Additionally, we aim to give researchers in other wide-ranging research fields incentives to start new studies into the development of new protein refolding methods.

2 Progress in refolding systems and physical conditions

2.1 Methods for removing denaturants

In dilution-based refolding, at the initial point of dilution, denatured proteins are exposed to the aggregation-promoting environment where the denaturant concentration around the proteins is rapidly reduced before each denatured protein has sufficiently separated from each other (Fig. 2A) [10, 16]. Under such an environment, denatured proteins immediately aggregate, especially at the high initial protein concentration. This environment is derived from the difference in the speeds of passive diffusion between small-molecule denaturants and relatively large proteins. Therefore, mixing methods and devices were studied to rapidly decrease local high concentrations of denatured proteins [17]. Furthermore, to increase the system productivity without increasing the initial protein concentrations, fed-batch and continuous dilution were extensively examined [18, 19]. Recently, laminar flow in microfluidic chips was employed to simultaneously control each concentration of denaturants and denatured proteins [20]. Although this microfluidic method is not suitable for industrial preparative refolding owing to its

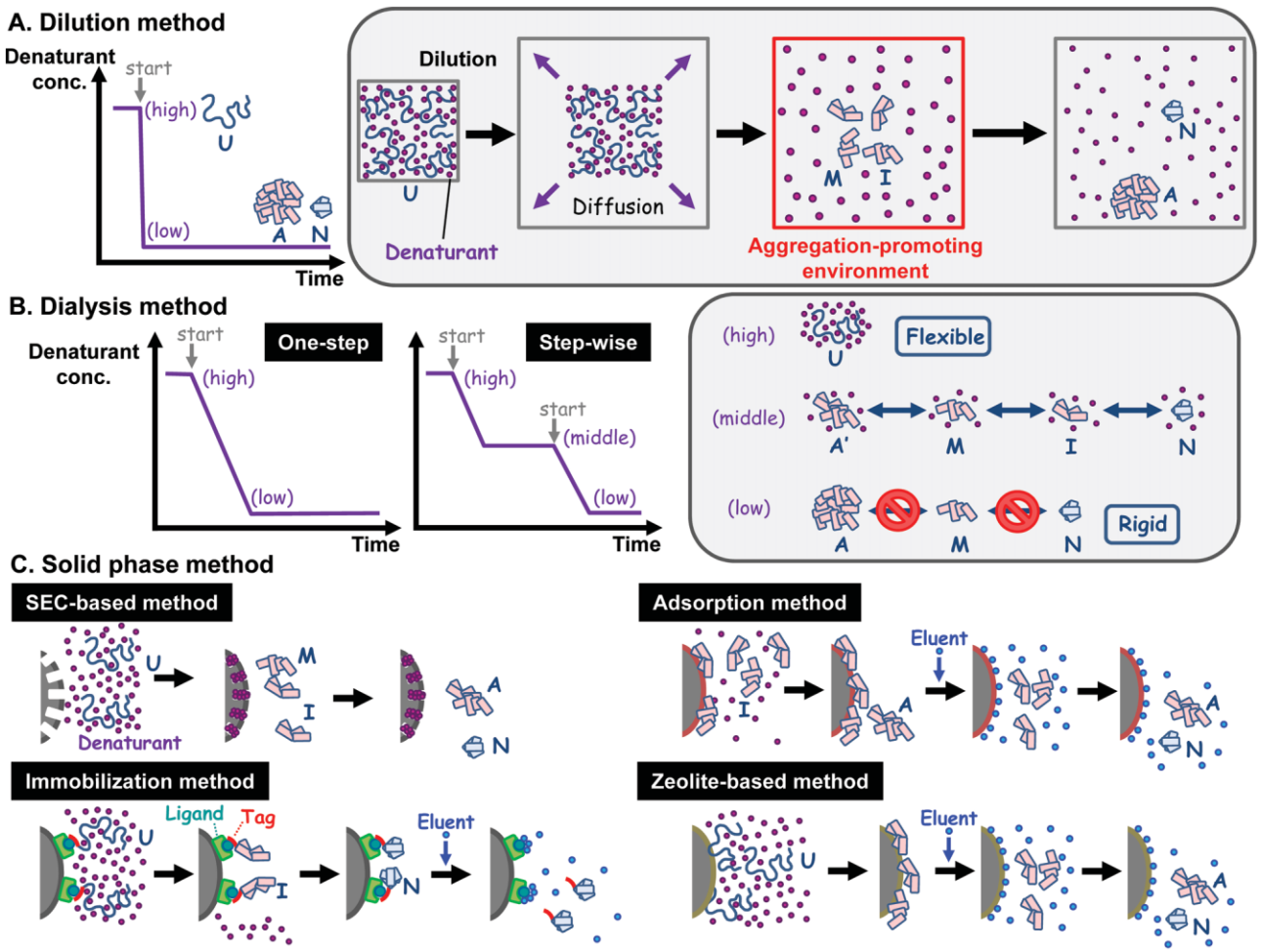

Figure 2. Schematic illustration of various methods for removing denaturants from solubilized protein solutions. (**A**) In dilution methods, the denaturant concentration around proteins rapidly declines through diffusion before the protein concentration has been attenuated adequately, leading to the aggregation-promoting environment where the local concentration of the refolding intermediate is high. (**B**) In dialysis methods, the denaturant concentration decreases gradually and uniformly. In a stepwise protocol, at the middle denaturant concentration, where the pathways to productive refolding or unproductive aggregation or misfolding are definitely selected, proteins can achieve equilibrium, whereas they transiently go through in a one-step protocol. (**C**) In solid-phase methods, various kinds of matrices aid refolding in each different mode: In SEC-based methods, denaturants are captured with porous matrices; in immobilization methods, ligand-modified matrices trap tag-fusion proteins under denaturation conditions, and then assist in refolding by inhibiting aggregation through isolation of proteins on their surfaces; in standard adsorption methods and a zeolite-based method, denaturants are washed out by adsorbing denatured proteins on matrices and proteins are subsequently refolded after release from matrices with eluents. A, U, M, I, and N represent the state of protein structures, as described in Fig. 1.

minute scale, it may serve as a strong tool for protein refolding on the laboratory scale.

Dialysis represents a second option for the removal of denaturants [1–5, 21, 22]. In dialysis-based refolding, protein concentrations are near constant before and after refolding. Accordingly, the initial concentration of the denatured protein can be substantially reduced, compared with dilution methods at the same final protein concentration. Furthermore, dialysis does not create local high concentrations of denatured proteins in a refolding solution during the initial stages of refolding. These features are more advantageous for suppressing aggregation. In addition, stepwise dialysis leads to high refolding yields (Fig. 2B) [5, 22]. As the denaturant concentration decreases by dialysis, protein molecules become less flexible because of the formation of intra- or intermolecular interactions [5]. In this process, it is crucial to control the concentration of the residual denaturant because unproductive misfolded and aggregated proteins cannot be renatured to the native state once the concentrations of the denaturant decrease to a low level where proteins are too rigid to undergo structural rearrangement (Fig. 2B). With a sufficiently high, but not too high, concentration of denaturant, the refolding state of proteins can reach equilibrium in the stepwise dialysis approach, whereas proteins are transiently exposed to such a midpoint denatu-

rant concentration in the simple one-step dialysis approach. Accordingly, stepwise dialysis is more effective in the following case: an equilibrium that favors correct folding over misfolding can be achieved only at the midpoint concentration, and it takes a long time to reach such an ideal equilibrium. Stepwise dialysis has been successfully shown to lead to a higher protein yield in the "slow" refolding of immunoglobulin-folded proteins or other proteins with a number of disulfide bonds [22]. Thus, dialysis under a suitable solution condition is very effective for the refolding of difficult-to-refold proteins.

The third option is to remove the denaturants by chromatography or the employment of solid phases [23–30]. The porous solids were used for trapping denaturants in SEC-based refolding (Fig. 2C) [23, 24]. This chromatographic method has specific advantages in terms of ease of automation and simultaneous purification capabilities. However, similar to the simple dilution method, the aggregation-promoting environment, where the denaturant surrounding the protein is rapidly removed, forms at the top of column. To fix these problems, a denaturant gradient system is used in which a gradual buffer exchange is introduced [24]. Different from the SEC-based methods, solid-assisted methods, in which denatured proteins are trapped on matrices, have been reported [25–30]. The denatured proteins with affinity tags such as a hexa-histidine tag and a self-splicing protein tag were refolded on ligand-modified matrices [26, 27]. In these methods, denatured proteins are immobilized away from each other on matrices at high denaturant concentrations and then triggered to refold by exchange to a refold buffer (Fig. 2C). Accordingly, denatured proteins can be refolded without interactions with other proteins on the matrices. These immobilization methods are elegant and useful on the laboratory scale. However, in industrial processes, the costs of expensive proteases for cleaving affinity tags after refolding may be problematic. On the other hand, nonspecific adsorption on various matrices has also been employed in chromatographic refolding methods [28–30]. Some proteins were successfully refolded through ion-exchange chromatography [28] or hydrophobic-interaction chromatography [29]. In these methods, the sometimes problematic specific tag for adsorption is not required. Proteins are captured in their denatured states on matrix surfaces (Fig. 2C). This can complicate the refolding of the adsorbed denatured protein on the matrices and most proteins may actually begin to be refolded after release from the matrices. In addition, in many cases, proteins adsorbed on the matrices compete with aggregation when the concentration of the denaturant decreases. Thus, denatured proteins tend to aggregate both before adsorption and after release (Fig. 2C). Therefore, the elaborate optimization of buffer conditions, such as gradients of denaturants or eluents, is necessary to obtain high refolding yields [29]. Recently, zeolites, which are crystalline porous aluminosilica compounds, were reported as novel matrices for refolding [30]. Zeolites can strongly capture denatured proteins at high denaturant concentrations without the need for any tags (Fig. 2C). Consequently, protein aggregation was efficiently suppressed before adsorption, and then, high refolding yields were reported for some proteins, including a protein with disulfide bonds [30].

Other unique methods for removing denaturants were previously reported. Protein refolding was performed in nanoscale aqueous droplets of reversed micelles dispersed in bulk organic solvents [31]. In this refolding method, a single denatured protein was isolated in such an aqueous compartment with highly concentrated denaturants and the denaturant concentration was decreased successively by mixing with a large amount of the reversed micelles without a denaturant. Compared with simple dilution methods, the relatively high refolding yields achieved were probably because the intermolecular interactions between denatured proteins were inhibited due to the isolation effect of the reversed micelles. Similarly, the liquid–liquid two-phase extraction system was used to remove denaturants, while inhibiting interactions between denatured proteins [32]. Furthermore, an interesting method using an enzyme, urease, to decrease denaturant concentrations was also reported [33]. This enzyme can catalyze the hydrolysis of urea to produce NH_3 and CO_2. This method enables the slow and uniform decrease of the denaturant without the need for a large-volume refolding buffer. Accordingly, this enzymatic method has the same advantages as the dialysis method. Thus, although most of these recent works are only proofs of principle, such unique methods may provide a breakthrough in industrial refolding of difficult target proteins.

2.2 Physical conditions for high-yield refolding

Protein refolding is usually performed at low temperatures to reduce protein aggregation [34, 35]. At low temperatures, a number of proteins were reported to be unfolded [36] and several oligomeric proteins were dissociated [37]. These phenomena can be explained by the exposure of hydrophobic side chains to water following the dissociation of hydrophobic interactions. Since such hydrophobic interactions are entropy driven due to the release of the water bound to nonpolar groups, this contribution should be weaker because the entropy contribution to the Gibbs free energy, $T\Delta S$, decreases at lower temperatures [37]. Accordingly, in the refolding process, lowering the temperature leads to the suppression of aggregation and refolding. Therefore, to increase the refolding yields using the dilution method, a temperature-shift procedure was reported [35]. In this procedure, aggregation of denatured proteins was suppressed at extremely low temperatures when refolding was initiated and then a temperature jump was performed to enhance refolding. When the refolding process included a rapid transition to agglutinative refold-

ing intermediates, this procedure was effective in avoiding the off-pathway to unproductive aggregation [35].

Similar to low temperatures, high hydrostatic pressure was employed to effectively suppress unproductive aggregation in refolding processes [38, 39]. Intra- or intermolecular hydrophobic interactions are accompanied by an increase in volume, because of the formation of solvent-excluding cavities between the hydrophobic interfaces and the release of bound water [40, 41]. High pressure can compress the volume of systems and lead to the suppression of hydrophobic interactions. It was reported that moderate hydrostatic pressure affected aggregation without influencing the folding of a native protein [38, 39]. By optimizing conditions such as pressure, temperature, and additives in the buffer, a high-pressure-assisted refolding method could achieve extremely high refolding yields, almost 100%, and at higher protein concentrations than the simple dilution method [39]. Furthermore, this method has a significant advantage in refolding directly from inclusion bodies by omitting the solubilizing step. Although the equipment for sample pressurization is not ubiquitous in biological or bioengineering laboratories and not easy to scale to industrial levels, the high-pressure refolding approach is highly attractive.

High temperature was also reported to increase refolding yields in the dilution method [42]. In this method, refolding samples of chemically denatured and reduced proteins were heated for only 5 min at the beginning of refolding and then incubated at a low temperature. As a result, heating above the melting temperature of proteins could increase the refolding yield. The increase was explained as follows:

(i) high temperature destabilized the aggregation-prone intermediates trapped in local energy minima and productive intermediates could predominantly be formed; and

(ii) the rate of rearrangement of non-native disulfide bonds increased with heating. Currently, high-temperature-assisted refolding was only reported to be an effective approach for one protein. However, this method is simple and appears to be worth testing at least once for each target protein.

3 Progress in refolding additives

3.1 Stabilizers of native proteins and refolding enhancers

In most refolding systems, the competitive reaction between productive refolding and unproductive aggregation occurs in aqueous solution. Therefore, the solution conditions of the refolding buffer are critical to improve refolding yields. The basal parameters of buffered solutions, such as pH, ionic strength, and buffering agent, affect protein refolding [12]. Therefore, when a new target protein is first refolded, such parameters must be routinely optimized. On this basis, to increase the refolding yields, additives were employed to create an ideal environment where the rate of refolding is increased, the aggregation rate is decreased, and the native folded protein is stabilized rather than undergoing a reverse transition to intermediate states. Currently, various additives have been reported as stabilizers of the native state of proteins, enhancers of refolding, and inhibitors of aggregation. These effective additives were found in a broad repertoire of molecular species, such as small synthetic or natural compounds, synthetic polymers, and proteins.

Over a century ago, Hofmeister found that the nature of co-existing salts changed the solubility of a protein [43]. On the basis of salting-in or -out effects, ions in a series can be qualitatively ranked and ions that tend to solubilize and denature proteins are classified as chaotropes. Conversely, ions categorized as kosmotropes agglutinate proteins and stabilize protein structures [44]. In refolding methods, the kosmotropic anion, ammonium sulfate, was employed as a stabilizer [45, 46], and such a salt decreased the rate of protein unfolding from the native state [45]. Similarly, sugars, polyols, betaines, and hydrophilic polymers were used to stabilize the folded protein and contribute to an increase in correctly folded protein yields [46–48]. The stabilizing effects of these additives were often explained by their preferential exclusion from protein surfaces, which thermodynamically led to the reduction of protein surface exposed to the solvent through unfavorable interactions between protein surfaces and additives [49] (Fig. 3). However, such stabilizers do not only increase the refolding yield, but simultaneously enhance aggregation. Accordingly, stabilizers have always been used in combination with aggregation inhibitors [46–48].

The refolding of difficult-to-refold proteins often includes the formation of more than one disulfide bond. In such cases, to increase the rate of oxidation and to break the non-native disulfide bonds, oxidized and reduced glutathione (GSSG, GSH, respectively) are commonly used as "disulfide-shuffling" reagents [1–4, 12, 50, 51]. In this system, GSSG works as an oxidizing agent to promote disulfide bonds between the thiols of two cysteine side chains in the protein. Therefore, if only GSSG is added, based purely on statistics, both correct and incorrect disulfide bonds are formed by random pairing of multiple cysteine residues. On the other hand, the addition of GSH can break incorrect disulfide bonds and trigger a cycle of reduction and oxidation steps, which finally result in the formation of disulfide bonds that give the correct protein fold. This is because a correct disulfide bond is stabilized by the free energy derived from the formation of the native conformation [12]. Other thiol–disulfide pairs, such as cysteine and cystine, also exerted similar effects [12, 51]. These disulfide-shuffling reagents can drastically increase refolding yields by increasing the yield of correct disulfide bonds and the rate of refolding when compared with oxidative refolding with only dissolved oxygen [50, 51].

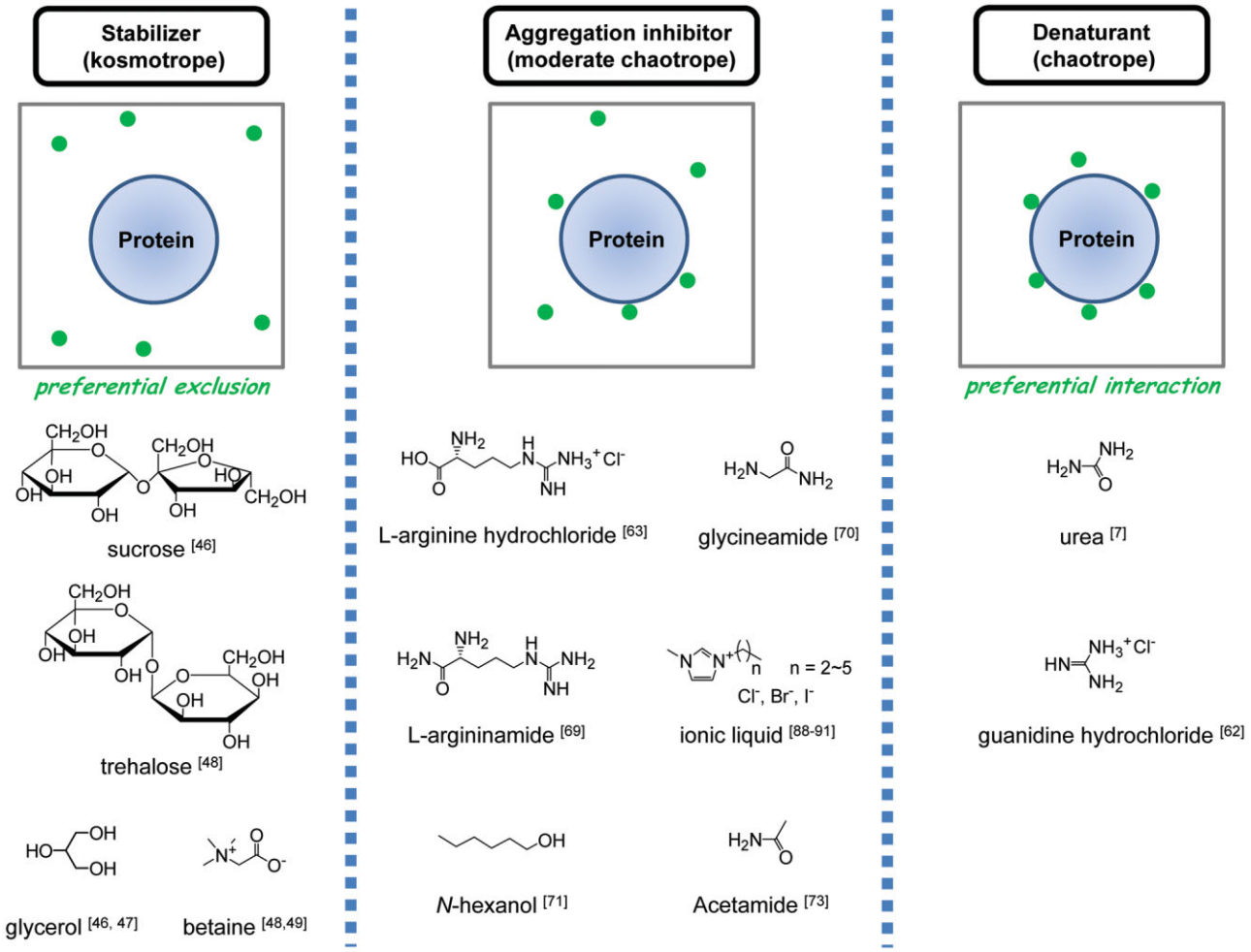

Figure 3. Various modes of interaction between a protein and refolding additives and the chemical structures of typical additives in each category. Modified with permission from Elsevier [64].

To enhance refolding in vitro, foldases that assist the folding of nascent proteins in vivo were employed. The employed foldases were categorized as protein disulfide isomerase (PDI) [13, 52, 53] and peptidyl prolyl *cis–trans* isomerase (PPI) [53, 54]. As part of the former enzymes, Dsb families, such as DsbA and DsbC, catalyzed disulfide-shuffling in vivo and in vitro. In detail, these PDIs in the oxidized form can catalyze disulfide formation by reacting with the thiol group of reduced target proteins, and those in the reduced form can trigger shuffling by attacking the incorrect disulfide bonds [13]. These foldases increased the refolding yield of a protein with multiple disulfide bonds. On the other hand, both subclasses of PPIs, cyclophilin and FK506 binding protein (FKBP), accelerated productive refolding and increased the yield of native protein [54]. They can catalyze the rate-limiting isomerization of Xaa–Pro (Xaa: any residue) peptide bonds.

In biomimetic chemistry, small synthetic reagents with PDI-like function were developed to improve the refolding rate [55–61]. At the active site of many PDIs, a common sequence motif CXXC (C: cysteine, X: any residue) is conserved and the one water-exposed thiol group has a low p*K*a value and high nucleophilicity at neutral pH, which leads to rapid nucleophilic attack on disulfide bonds of folding intermediates. To mimic such nucleophilicity, reducing reagents with low p*K*a values, such as aromatic thiols [55], selenoxides [56], and selenoglutathione [57], were developed and successfully led to the rapid formation of multiple disulfide bonds. More directly, peptides consisting of the CXXC motif of disulfide oxidoreductases were also employed for oxidative refolding [58]. Furthermore, a synthetic small-molecule dithiol with the same p*K*a value and reduction potential as the CXXC motif of PDI enhanced the rate of disulfide shuffling [59]. Recently, to mimic the hydrophobic regions around the active sites of PDI, hydrophobic alkyl chain modified cyctamines were developed [60]. By these biomimetic approaches, the function of foldases could be substituted with small-molecule mimics. Since the use of foldases has the disadvantages of costly production and

instability, these mimics are useful for industrial refolding as low-cost and stable substituting reagents [61].

3.2 Aggregation inhibitor

To inhibit aggregation, small-molecule additives are frequently employed because of their low cost and ease of removal after the refolding process [1, 5]. In particular, chaotropes, such as urea and guanidine hydrochloride (GdnHCl), are generally used at relatively low concentrations from 0.5 to 2.0 M [7, 62, 63]. Such chaotropes were originally employed as denaturants in refolding processes. At high urea and GdnHCl concentrations, protein aggregates are effectively dissolved and protein unfolding also occurs because these chemicals disrupt both intra- and intermolecular interactions of proteins. The mechanism underlying chaotropic effects remains the subject of some debate [44, 64]. Recently, the most widely accepted explanation is the preferential interaction of chaotropes with protein surfaces (Fig. 3). Such interactions stabilize the exposed surface of the protein, resulting in a decrease in the free energy of unfolding and an increase in the free energy of aggregation [64]. In a standard refolding method, chaotrope concentrations are drastically decreased to initiate the protein refolding process; however, in some cases, high refolding yields were obtained by using chaotropes at nondenaturing concentrations [7, 62]. Under such positive conditions, the aggregation rate was remarkably decreased compared with the refolding rate. Although the mechanism by which aggregation is selectively suppressed remains unclear, the weak interaction of chaotropes with the hydrophobic surface of proteins may create a kinetic situation where only intramolecular hydrophobic interactions are formed.

Rudolph and Fischer [65] first reported the function of L-arginine hydrochloride (L-ArgHCl), a well-known amino acid based chaotropic reagent, on the suppression of aggregation and enhancement of protein refolding. Subsequently, the ability of arginine to increase refolding yields was tested with a variety of proteins and was effective over the concentration range of 0.4 to 1 M [63, 66, 67]. Currently, L-ArgHCl is the most commonly used additive. Compared with GdnHCl, L-ArgHCl, which has the same guanidinium moiety, is a superior aggregation inhibitor for the refolding process because its protein denaturing effect is more moderate (Fig. 3). As described above, such a selective effect of chaotropes is explained by several theories and many studies describing the mechanism of L-ArgHCl were recently reported [63, 67, 68]. Among them, the "gap effect theory" suggests that additives larger than water, which do not affect the folding of isolated proteins, can selectively increase the free energy of intermolecular protein–protein association [67]. This theory appears to clearly account for the observed effects of various aggregation inhibitors.

Other moderate chaotropes have been reported as effective aggregation inhibitors for protein refolding (Fig. 3) [69–75]. Amino acid derivatives, such as L-argininamide and glycineamide, increased the refolding yields of some proteins more than L-ArgHCl [69, 70]. Short-chain alcohols and polar organic solvents exerted a similar refolding-promoting effect on protein refolding [71–73]. Although their chaotropic effects have not been discussed yet, they presumably operate through the same mechanism. Thus, many small-molecule chaotropes can assist in preparing suitable solution conditions for protein refolding and some of them are already commonly used in both laboratory and industrial settings. However, although their cost is relatively low relative to other molecular species, on the industrial scale, amino acids and their derivatives are very costly [3]. Therefore, more inexpensive or inexpensive, recyclable chaotropes are required.

Additives that bind more strongly to protein surfaces than chaotropes were also reported as aggregation inhibitors for refolding. In this category, detergents are most commonly used [71, 74–86]. Currently, various kinds of detergents, such as cationic, anionic, zwitterionic, and nonionic detergents, within a nondenaturing concentration range can prevent aggregation and enhance refolding yields [71, 74–77]. Such suitable refolding conditions were assumed to require the formation of mixed micelles consisting of proteins and detergents, and the relationship between the refolding yields and their critical micellar concentrations was discussed [74]. Furthermore, protein–detergent interactions were extensively studied by indirect and direct methods [64]. However, in many cases, detergents inhibit not only aggregation, but also refolding due to strong interactions between detergents and refolding intermediates [79, 80]. Therefore, employment of detergents often leads to low refolding yields and extremely long refolding times. In addition, different from salts and chaotropic reagents, detergents are difficult to remove from products by dialysis or gel filtration due to the formation of micelles and their strong interactions with protein surfaces. Therefore, in subsequent steps, adsorbents for detergents, such as reverse-phase chromatography or stripping reagents, are required.

Similarly, other compounds, such as cyclodextrin derivatives [81, 82], polymers [71, 82–84], and sulfobetaine [85], which can bind to hydrophobic protein surfaces, increased refolding yields. In the refolding processes, these additives must meet two conflicting requirements: (i) they are required to attach to the hydrophobic surface to inhibit intermolecular interactions between proteins; and (ii) they are simultaneously required to detach from the protein surface, so that inhibition of intramolecular interactions does not occur. Therefore, their positive effects are limited to a narrow optimal concentration where the additives moderately interact with exposed protein surfaces. Furthermore, depending on the target protein, such positive effects are often not exerted, even at the optimal

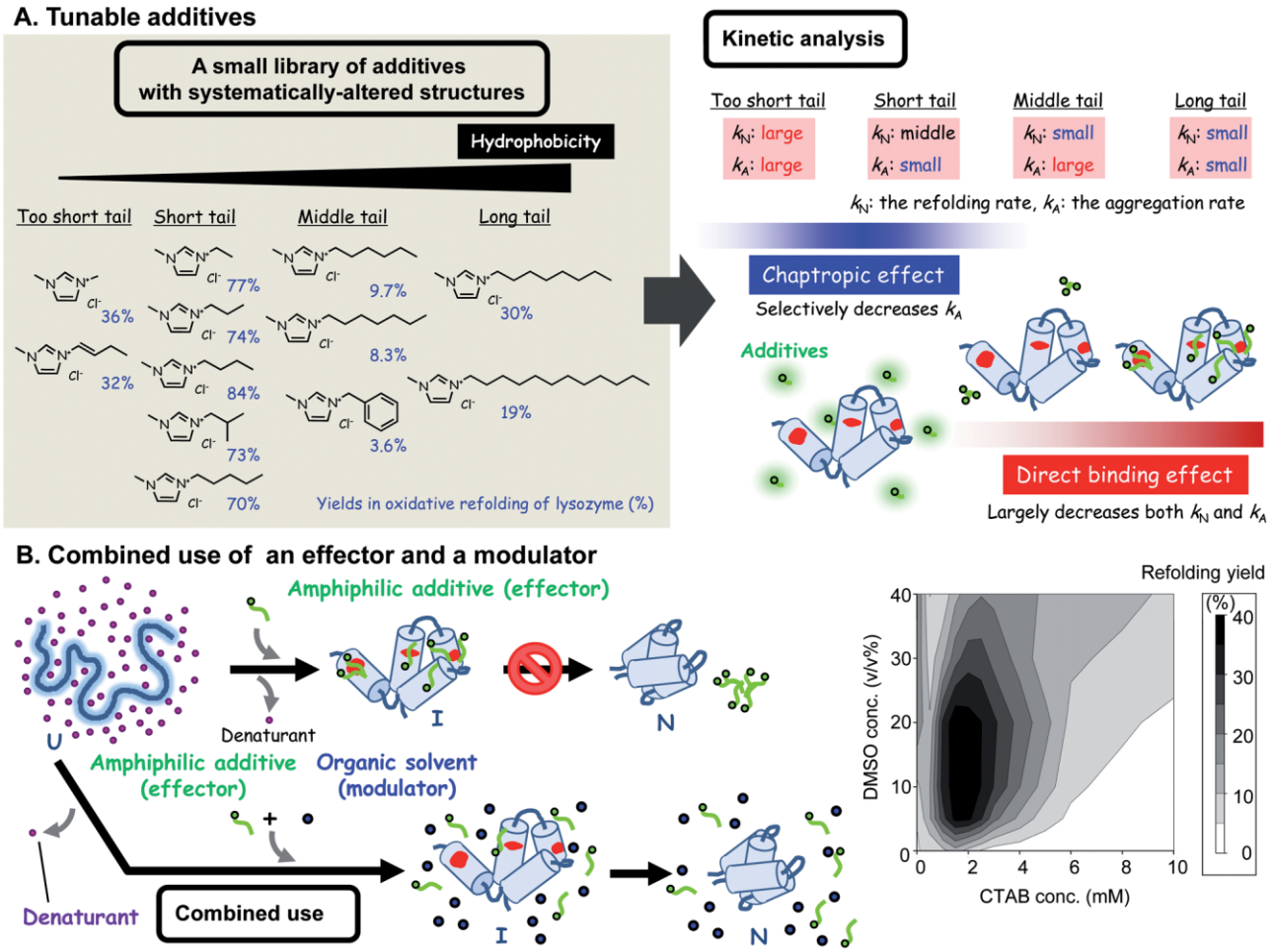

Figure 4. Two modes of tunable additives for protein refolding. (**A**) A small library of chemical additives with systematically altered structures was easily synthesized as tunable additives. By kinetic analysis of the refolding process, additives with a short hydrophobic tail exerted a positively chaotropic effect on the oxidative refolding of lysozyme, and on the other hand, those compounds with a long tail worked effectively as detergents [89]. Thus, alteration of the tail length can control the rates of refolding and aggregation. (**B**) Schematic illustration of the combined use of a detergent and an organic solvent. The organic solvent modulates the interaction between proteins and detergents, resulting in a synergistically positive effect on the refolding yield. The data was obtained from [92]. U, I, and N represent the state of protein structures, as described in Fig. 1.

concentration. In most previous studies, commercially available amphiphilic compounds were nonsystematically employed as aggregation inhibitors by trial-and-error approaches. Consequently, optimization of a refolding solution needs significant time. Recently, "tunable" additives, the derivatives of which have systematically altered hydrophobic moieties that were easily prepared, were reported [86–91]. In this approach, the relationships between chemical structures and effectiveness at increasing the refolding yield were assessed and then the manner in which the chemical substructures confer suitable properties on protein refolding could be understood. Ionic liquids with various hydrophobic tails, cationic heads, and counteranions were investigated and those with both a short alkyl chain and a hydrophilic anion were excellent refolding additives due to their moderate chaotropic effects (Fig. 4A) [88–91]. Conversely, ionic liquids with a long alkyl chain only worked effectively over a limited concentration range, in a similar manner to detergents. Ionic liquids with an average (mid-length) alkyl chain length or a benzyl group had no positive protein refolding effects (Fig. 4A) [89]. Thus, tuning the properties of additives is a successful strategy to obtain tailor-made additives for each target protein. This strategy can also be expanded to a variety of additives, the mechanisms of which for assisting protein refolding are well known and may potentially result in the rational design of a solution environment for the refolding of a target protein.

To more easily modulate the properties of aggregation inhibitors, the combined use of an effector and a modulator were reported (Fig. 4B) [92]. As described above, refolding additives of detergents often inhibit productive refolding because such additives strongly bind to the hydrophobic surfaces of refolding intermediates. Accord-

ingly, to modulate protein–detergent interactions, various organic cosolvents were added with detergents, and consequently, both the refolding and aggregation rates were controlled, according to the log*P* values and the concentration of the cosolvents. Moreover, the refolding yields were synergistically increased by employing polar cosolvents at a moderate concentration with various detergents [92]. Such a combined use of conventional additives is inexpensive and easy to perform because we do not need to find or synthesize new, effective additives. Thus, tuning the effect of conventional aggregation inhibitors with modulator agents may expand the application ranges of existing refolding additives.

A negatively charged long polymer aided refolding of cationic proteins by interacting with the protein surface through an electrostatic interaction [93]. It was speculated that the polymer could shield the hydrophobic surfaces of refolding intermediates because of steric hindrance; thus suppressing protein aggregation. Different from standard aggregation inhibitors, such polymers do not interact with the hydrophobic patches located on refolding intermediates required for packing of the tertiary structure. Accordingly, these polymers avoid the conventional conflicting requirements of aggregation inhibitors, and therefore, unproductive aggregation of refolding intermediates can be specifically suppressed without inhibition of productive refolding. In addition, such charged polymers are removed from the protein surface by changing the pH or employing ion-exchange chromatography after refolding, and thus, represent promising tools for both laboratory- and industrial-scale refolding.

The two conflicting requirements for aggregation inhibitors of protein refolding are overcome by using molecular machines, named molecular chaperones in vivo [94]. Chaperonin GroEL, which is the most well-known molecular chaperone, can sequentially capture and release unfolded substrate proteins to assist folding. In detail, the double-ring GroEL tetradecamer encapsulates substrate proteins in the central cavity when capped by the GroES heptamer in an ATP-dependent manner. In this GroEL/GroES system, the substrate binding site of GroEL alters the exposure of its hydrophobic surface through a conformational change in coupling to both ATP hydrolysis and competitive binding of GroES. This dynamic allosteric alteration of GroEL enables the captured protein to be released, and additionally, a released, unfolded protein is isolated in each cavity of the GroEL/GroES complex to fold without associating with other unfolded proteins. Application of such chaperonins to in vitro protein refolding has been extensively studied and chaperonins from various bacteria increased the refolding yields by suppressing unproductive aggregation of target proteins [95, 96]. In addition, only the monomeric polypeptide-binding domains of GroEL increased the refolding yield; however, the chaperone activity of this GroEL was much lower than the activity of the full GroEL/GroES system [97, 98]. Thus, such dynamic allosteric properties and the cavity for isolation are not essential for chaperone activity, but the two effects of the GroEL/GroES system are certainly effective at increasing the refolding yield.

Similar to foldase mimics, the functions of chaperones were challenged by substitution with synthetic compounds [16, 80, 98–107]. To mimic the allosteric properties of chaperones, Rozema et al. [16, 80] established artificial chaperone-assisted (ACA) refolding, which involves a two-step dilution procedure (Fig. 5A). In the first step, the denatured protein solution is diluted with a buffer containing detergents to prevent aggregation by forming protein–detergent complexes. In the second step, the protein–detergent complex solution is diluted with a buffer containing detergent strippers, such as β-cyclodextrin. The first step mimics the capture of an unfolded protein with GroEL and the second step leads to the release of the protein, as GroEL allosteric functions. The ACA refolding approach is effective in refolding systems for a large variety of proteins [16, 80, 99, 100]. Furthermore, various detergent strippers of oligomeric, polymeric sugars, and cyclodextrin-modified polymers are useful for this method [100–103]. In ACA refolding methods, the detergent strippers effectively remove the detergents, so that the dilution ratio in the second refolding step can be substantially lowered compared with conventional simple dilution methods (Fig. 5A). Accordingly, similar to the dialysis method, the protein concentrations at the start of refolding can be drastically reduced, resulting in the suppression of unproductive aggregation [16].

Smart polymer-assisted refolding was reported as a method that mimicked the allosteric chaperone function [104]. Temperature-responsive polymers, such as poly(*N*-isopropylacrylamide) (PNIPAAm), can alter their hydrophobicity as a function of temperature: their hydrophobicity drastically increases above their low critical solution temperature (LCST). Accordingly, for the initial dilution, the refolding mixture was incubated at a higher temperature than that of the LCST to inhibit aggregation through the formation of strong polymer–protein complexes. Refolding was then started by releasing the protein from the polymer–protein complexes by lowering the temperature [104]. Recently, an enzyme-responsive surfactant was employed as a changeable aggregation inhibitor for protein refolding [105]. This amphiphilic surfactant was reported to release captured refolding intermediates as enzymatically polymerized, more hydrophilic forms; thus yielding native protein without unproductive aggregation. On the other hand, the function, like the isolation effect observed for chaperonins, was exerted by nanogels of self-assembled cholesterol-bearing pullulan [106, 107]. Refolding intermediates were spontaneously captured into nanogels through hydrophobic interactions and the captured proteins were effectively released in their refolded native form upon dissociation of nanogels in the presence of cyclodextrin [106] or upon al-

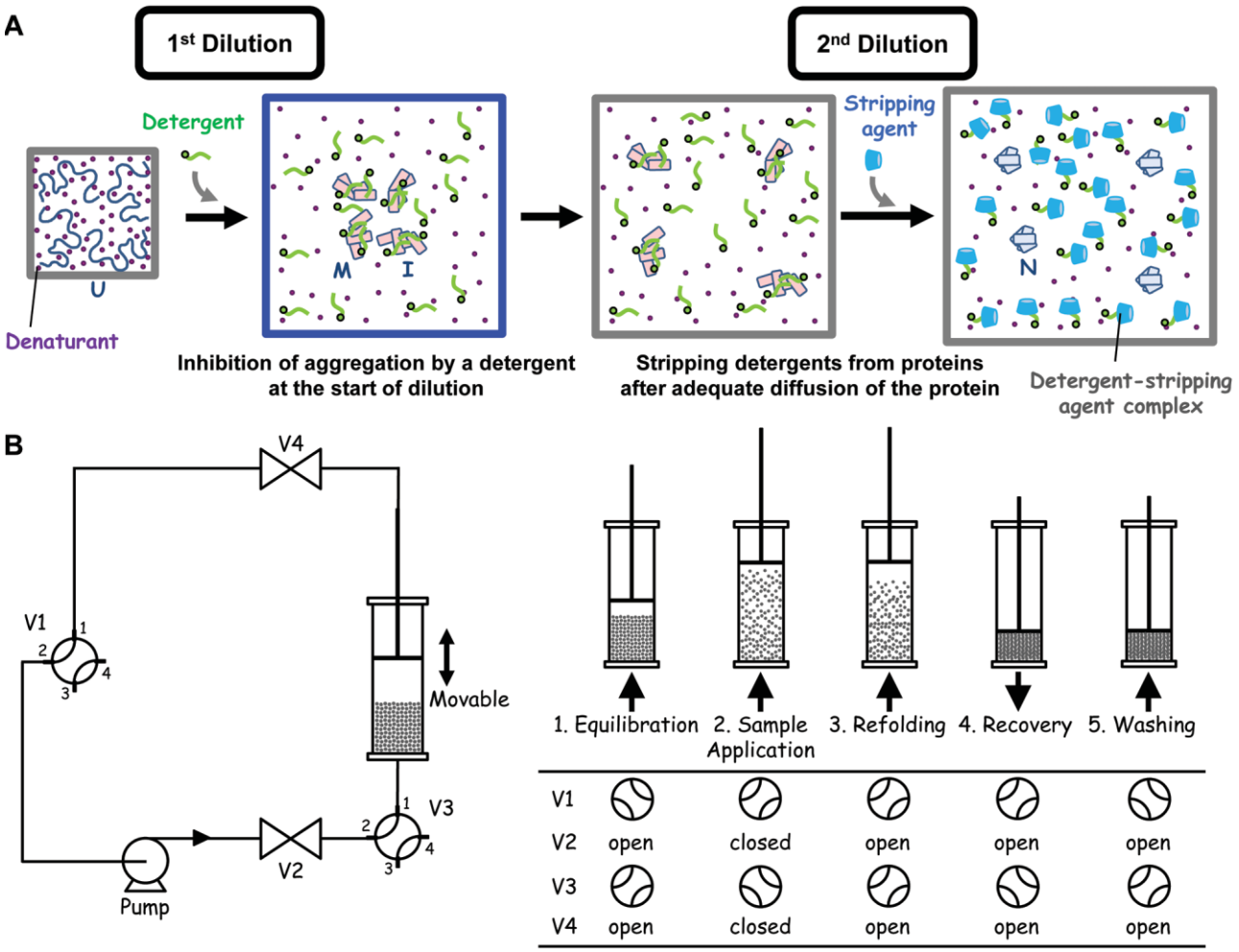

Figure 5. Schematic illustration of ACA refolding and its application to an expanded-bed column system. (**A**) In the ACA method, protein aggregation following the initial high dilution of the denaturant concentration is suppressed by adding detergents (the first step) and, once the protein has adequately diffused, the detergent is removed from the protein surfaces by the addition of detergent stripping agents, thereby resulting in a high yield of refolded protein (the second step). U, M, I, and N represent the state of protein structures, as described in Fig. 1. (**B**) Experimental setup of the expanded-bed refolding system based on the solid-phase ACA method (left). The protein–detergent complex solution was applied to a cyclodextrin-bead-loaded column and refolding through the removal of detergents from the complex was performed by circulating the solution. Operational modes of the column and the valves are shown (right). By repeating steps 1 to 5, refolding can be performed continuously. Reprinted with permission from Elsevier [101].

teration of the hydrophobicity of the nanogel by photostimulation [107]. This nanogel-assisted refolding successfully increased the refolding yields of some proteins compared with the spontaneous refolding system. Thus, in such biomimetic approaches, several artificial chaperones based on various materials were successfully developed and could effectively suppress aggregation of refolding intermediates by capturing proteins in an aggregation-promoting environment and releasing the protein into a suitable environment for refolding.

3.3 Application of refolding additives

In many cases, novel refolding additives are initially evaluated for their efficacy in the simple dilution methods. However, most of them are compatible with other refolding systems. Disulfide-shuffling reagents and chaotropic additives, for example, have been routinely used in dialysis- and SEC-based methods [22, 24]. Molecular chaperones were also applied to a reversed micelle system and SEC-based refolding; thus increasing the refolding yield [108, 109]. Recently, the ACA method was actively reported to be combined with other techniques [110, 111]. The synergistic effects of the combined use with the low-temperature method were studied [110]. In addition, to decrease the initial protein concentration, the ACA approach was employed in microfluidic-aided refolding, resulting in the effective suppression of aggregation at the mixing point [111]. On the other hand, many studies involving the combined use of refolding additives, such as

an aggregation inhibitor and a protein stabilizer, have also been reported for more than 15 years [112]. The combined use of two foldases and a molecular chaperone represents very famous work in this research field [12]. In addition, other combinations such as ACA additives and chaotropes [113], and a molecular chaperone and polymers [114], have been reported. Thus, the combined use of the reported methods and additives is a promising approach to further increase the refolding yield, especially when the two aspects complement each other. Because many combinations still remain untested, there is the potential for the emergence of a surprising number of valuable systems from such a combinatorial approach.

Some refolding additives have been introduced to a solid phase for the following reasons: (i) they can be stabilized through immobilization on matrices; (ii) insoluble additives are easy to remove after refolding; (iii) they are easy to reuse; and (iv) the advantages of chromatographic refolding, such as ease of automation, simultaneous purification capabilities, and downsizing of equipment requirements, may be available. Foldases and a molecular chaperone were immobilized on matrices and used in refolding chromatography [13, 53, 98, 115]. Similarly, small-molecule thiols were immobilized on microspheres and employed as a solid-phase PDI mimic [116, 117]. Additionally, solid-phase ACA refolding methods were actively studied by using various cyclodextrin polymer beads or cyclodextrin-modified microspheres [101, 102, 118, 119]. Solid-phase ACA refolding was successfully performed in an expanded-bed column system, which was easy to scale up by using commercially available devices (Fig. 5B) [101]. By increasing the column/loop volume ratio, almost the same refolding yields as that of a liquid-phase system were obtained in such a column system. The loaded cyclodextrin polymer beads could be reused by simple washing with water. Thus, the application of refolding additives to solid-phase systems represents a strong tool for protein refolding on the industrial scale.

4 Conclusion

In the present post-genome era, a rapid and inexpensive method for protein production is becoming increasingly important in various research fields. In this context, the inclusion-body-based production system is attractive because of the high protein expression yields and the efficiency of upstream processes. However, this production system often has significant drawbacks related to the refolding step. To date, many methods have been developed to improve the refolding yield. In particular, effective refolding additives have been explored to create suitable conditions where productive protein refolding dominates over unproductive protein aggregation in the kinetic competitive reaction. To increase the refolding rate, excellent reductive/oxidative reagents, which mimic naturally occurring foldases, have been developed and their effects are potentially universal in oxidative refolding of any proteins. On the other hand, many aggregation inhibitors were also reported, but versatile chemical tools for any target protein have not been found yet due to the infinite variability of protein surfaces. Therefore, recently, tunable synthetic additives and the combined use of an effector and a modulator were developed for "tailor-made" refolding. Moreover, understanding the mechanism by which these systematically altered additives have an effect on protein refolding and aggregation enables rational selection and design of refolding additives for a novel target.

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Therefore, progress in analytical methods to elucidate the effect of protein-protein and protein-additive interactions on refolding processes is also extremely important [120]. In addition, refolding additives that mimicked the function of molecular chaperones were actively studied because chaperones were known to aid folding of a wide variety of proteins. Furthermore, some effective combinations of refolding additives and conventional techniques increased the refolding yields, and the introduction of refolding additives to solid-phase techniques successfully led to suitable methods for industrial refolding. Although the majority of positive results obtained from protein refolding studies were obtained using several model proteins, the present emerging method based on refolding additives is expected to alter the "troublesome" refolding process to a routine one in the laboratory and the manufacturing area in the future.

The authors declare no conflict of interest.

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