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Physical–chemical features of non-detergent sulfobetaines active as protein-folding helpers☆

Nicole Expert-Bezançon[a,1], Thierry Rabilloud[b], Laurent Vuillard[c,2], Michel E. Goldberg[a,*]

[a]*Unité de Repliement et Modélisation des Protéines, Institut Pasteur, Paris, France*
[b]*DBMS/BECP, EA 2943, CEA Grenoble, France*
[c]*Institut Laue Langevin, Grenoble, France*

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Abstract

Some non-detergent sulfobetaines had been shown to prevent aggregation and improve the yield of active proteins when added to the buffer during in vitro protein renaturation. With the aim of designing more efficient folding helpers, a series of non-detergent sulfobetaines have been synthesized and their efficiency in improving the renaturation of a variety of proteins (*E. coli* tryptophan synthase and β-D-galactosidase, hen lysozyme, bovine serum albumin, a monoclonal antibody) have been investigated. Attempts to correlate the structure of each sulfobetaines with its effect on folding revealed some molecular features that appear important in helping renaturation. This enabled us to design and synthesize new non-detergent sulfobetaines that act as potent folding helpers.

Keywords: Physical–chemical features; Non-detergent sulfobetaines; Protein-folding helpers

1. Introduction

Recombinant DNA technology nowadays enables researchers and biotechnologists to easily clone a gene of interest and express it in a variety of cell types. The resulting proteins are, however, often obtained in a non-native form that fails to exhibit the desired biological activity, particularly when high expression levels are reached. The formation of misfolded protein, frequently present as insoluble aggregates (inclusion bodies), is currently the most severe bottle-neck in the mass production of natural or recombinant proteins for research as well as industrial purposes. It imposes that procedures should be developed for solubilizing the aggregates and renaturing the polypeptide chains. Because the efficiency of renaturation depends on the kinetic partitioning between a productive monomolecular folding reaction and a multimolecular abortive aggregation reaction [17,21], in vitro folding procedures are often efficient only at low protein concentrations (fractions

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*Corresponding author. Tel.: +33-1-45-68-83-86; fax: +33-1-40-61-30-43.

E-mail address: goldberg@pasteur.fr (M.E. Goldberg).

[1] Present address: Unité de Biochimie Structurale, Institut Pasteur, Paris, France.

[2] Present address: AVIDIS, Biopole Clermont Limagne, Saint Beauzire, France.

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of mg ml^{-1}) where multimolecular reactions are slowed down. Therefore, enormous reaction volumes are needed to treat industrial quantities of proteins. Hence the important impact of the renaturation step on the investment and running costs of recombinant protein production. These considerations account for the growing interest in low molecular weight additives that prevent aggregation during protein renaturation.

The first additives used in such a way were urea and guanidinium chloride [17]. Since then, many others have been investigated and used successfully for renaturing a variety of proteins. They have been the topic of a recent review [13]. Among these additives, a not yet popular but very useful family of molecules are non-detergent sulfobetaines (NDSBs), several of which have been shown to dramatically increase the yield in native protein during the oxidative folding of reduced hen lysozyme, and upon renaturation of *E. coli* β-D-galactosidase [9]. These molecules were shown to prevent protein aggegation by interacting with early folding intermediates [19]. Since these early studies, NDSBs have helped in the renaturation of several proteins (e.g. in Chong and Chen [2] and in Ochem et al. [16]). Yet, only a limited number of proteins, and very few molecules of the NDSB family have been investigated. Hence our interest in extending our initial studies to other NDSBs and proteins.

In the present report, we describe the effect of SB256 (the most potent of the previously studied NDSBs) on the renaturation of the β_2 subunit of *E. coli* tryptophan synthase, of reduced bovine serum albumin, and of a murine monoclonal antibody. We also report the effects of 11 different NDSBs on the oxidative renaturation of reduced hen lysozyme and on the refolding of the *E. coli* tryptophan synthase β_2 subunit. On the basis of structural features shared by the best NDSBs, we designed, synthesized and tested two new 'optimized' NDSBs whose properties as folding helpers will be reported.

2. Materials and methods

2.1. Enzymes and chemicals

Hen egg white lysozyme was purchased from Roche Biochemicals and bovine serum albumin from Sigma. Both were used without further purification. Monoclonal antibody mAb164 was prepared as described previously [4,6] and the β_2 subunit of *E. coli* tryptophan synthase was prepared and activated according to a previously published method [11]. Rabbit anti-mouse immunoglobulin antibodies were from BioSys. All other chemicals were reagent grade, commercially available compounds.

2.2. NDSB synthesis

NDSBs (except SB201 which was purchased from Fluka) were synthesized as previously described [18]. Briefly, the corresponding tertiary amines, dissolved at 1 M in 1,2 dichloroethane, were heated up to mild reflux. A molar equivalent of propane sultone, pre-dissolved in dichloroethane, was added dropwise. A vigorous reaction took place, and heating was discontinued in order to maintain the reaction under control. Refluxing was continued for 4 h to overnight depending on the reactivity of the amine. The reaction mixture was allowed to cool. The product precipitated directly from the reaction mixture and was collected by filtration. After rinsing with acetone, the product was dried. The crude product was purified by recrystallization from water–methanol or ethanol, or ethanol–acetone.

2.3. Tryptophan synthase β_2 subunit unfolding, renaturation and assay

Crystals of β_2 were dissolved (approx. 12 mg ml^{-1}) in buffer containing 0.1 M Tris–HCl at pH 7.8, 2 mM EDTA, 5 mM DTT and 4 M guanidinium chloride, and dialyzed overnight at 4 °C against the same buffer, conditions sufficient to fully unfold β_2 [20]. The absorption spectrum of the dialyzed solution was recorded, using the dialysis buffer as a blank. The protein concentration was determined from the absorbance at 280 nm, using as-specific extinction coefficient $\varepsilon_{280} =$ 0.58 mg ml^{-1} cm^{-1}. [14]. The solution was diluted to 10 mg ml^{-1} with dialysis buffer. Aliquots of 10 μl were rapidly diluted into 190 μl of renaturation buffer [0.1 M Tris–HCl (pH 7.8), 2 mM EDTA, 5 mM DTT and 50 μM pyridoxal-5′-

phosphate supplemented with concentrations of NDSB ranging between 0 and 1.8 M in 0.2 M steps] under vigourous mixing in a Vortex mixer as described previously [8]. The solution was incubated at 20 °C for 4 h. Of the protein solution 50 μl were then added to 0.950 ml of assay mixture and the enzymatic activity determined as described previously [5]. The specific activity thus obtained was expressed in percent as compared to the specific activity of native β_2 determined in the presence of guanidinium chloride and NDSB at the same concentrations as those present in the assay mixture after addition of the renatured protein. The stability of the native protein in the renaturation conditions was checked by measuring the specific activity of native β_2 before and after a 4-h incubation at 20 °C in renaturation buffer supplemented with 1.8 M NDSB and 0.1 M guanidinium chloride. No significant change in activity was observed, thus no correction for inactivation had to be applied.

2.4. Bovine serum albumin unfolding, renaturation and characterization

Bovine serum albumin was dissolved at a concentration of 100 mg ml^{-1} in 100 mM potassium phosphate (pH 7.5), 6 M guanidinium chloride and 60 mM DTT, and incubated for 2 h at 20 °C. Oxidative renaturation was initiated by either 20- or 100-fold dilution (under vigorous mixing as above) into 50 mM Tris–HCl (pH 8.5), 5 mM EDTA and 5 mM oxidized glutathione, eventually supplemented with SB256 concentrations ranging between 0 and 1.8 M in 0.2 M steps. After overnight incubation at room temperature, the samples were subjected to centrifugation at 14 000 rev min^{-1} during 45 min in a bench centrifuge. The protein concentration in the supernatent was measured by the method of Bio-Rad protein assay, using BSA as a standard and taking into account the contribution to the absorbance at 595 nm of the residual SB256 present in the assay, which should be kept below 60 mM.

Non-denaturing PAGE was carried out on 8–25% of non-denaturing polyacrylamide gels using the PhastSystem (Pharmacia) at 165 VAh. Staining was with Coomassie Blue.

2.5. Monoclonal antibody mAb164 unfolding, renaturation and assay

Pure mAb164 immunoglobulins (7.5 mg ml^{-1} in phosphate buffered saline) were diluted 7.5-fold with 100 mM Tris–HCl (pH 7.8) containing 2 mM EDTA, 6 M GuHCl and 30 mM reduced DTT. The mixture was incubated for 2 h at 4 °C. Of the denatured/reduced protein 10 μl was diluted by vigorous mixing as above with 190 μl of renaturation buffer [100 mM Tris–HCl (pH 7.8), 2 mM EDTA, 5 mM oxidized DTT] supplemented with the desired amount of SB256. Thus, the protein concentration in the renaturation mixture was 50 μg ml^{-1} and the oxidation/disufide exchange catalyst was 1.5 and 5 mM, respectively, of reduced and oxidized DTT. After overnight incubation at room temperature, the renaturation mixture was diluted 50-fold with Tris–HCl (pH 7.8) containing 5 mM oxidized DTT and 1.5 mM reduced DTT, resulting in a 1-μg ml^{-1} total protein concentration. Native mAb164 was also diluted with the same buffer to 1 μg ml^{-1}. The renaturation mixtures and the native antibody were tested for active antibody by ELISA as follows. The coating and washing buffers, as well as the general procedures, were as described previously [7]. ELISA plates (96 wells) were coated by adding 100 μl of β_2 (10 μg ml^{-1} in classical coating buffer) in each well followed by overnight incubation at room temperature. The plates were washed three times with washing buffer. Of the mAb164 solution to be assayed (renatured or native at 1 μg ml^{-1}) 100 μl were added per well and incubated for 30 min at room temperature. The wells were emptied and washed three times with washing buffer. Each well was then filled with 100 μl of rabbit anti-mouse immunoglobulin antibodies coupled to β-galactosidase diluted 2000-fold in washing buffer. The plate was left for 30 min at room temperature and washed three times with washing buffer. Each well was finally filled with 100 μl of galactosidase substrate (*O*-nitrophenyl-β-D-galactopyranoside) solution and incubated at room temperature. When sufficient color developed, the absorbance in each well was recorded using a Titertek ELISA-plate reader. It was verified that, at all concentrations of SB256

used in the renaturation, the residual SB256 in the antibody solution after the final 50-fold dilution did not interfere with the ELISA. The yield in active antibody was expressed, in percent, as the absorbance in the well containing the renatured antibody relative to the absorbance in the well containing the native antibody.

2.6. Lysozyme unfolding, renaturation and assay

The denaturation/reduction, renaturation and assay of lysozyme were conducted as described earlier [8] except for the renaturation buffer which contained the desired concentration of NDSB (ranging between 0 and 1.8 M in 0.2 M steps) and 3 mM (instead of 0.3 mM) oxidized glutathione. The inhibitory effect of the residual NDSB in the assay mixture was determined by measuring the activity of native lysozyme in the presence of the added NDSB at the same concentration, and was taken into account to determine the yield in native protein after renaturation. It was checked that incubation of native lysozyme during 4 h at 20 °C in the renaturation buffer with NDSB did not affect its activity.

3. Results and discussion

3.1. Effect of SB256 on the renaturation of bacterial and mammalian proteins

Five NDSBs had been investigated for their efficiency in helping the renaturation of reduced hen lysozyme and bacterial galactosidase [9]. For these two proteins, SB256 turned out to be the most efficient folding helper. This molecule was therefore chosen to test the effect of NDSBs as additives for improving renaturation of other proteins. Three proteins that exhibit severe folding difficulties in vitro were selected for these studies: (1) the β_2 subunit of *E. coli* tryptophan synthase, a homodimer with no disulfide bonds; (2) bovine serum albumin, a monomeric protein with 17 disulfide bonds and a free cystein; and (3) a murine monoclonal antibody (IgG), a heterotetramer with intrachain as well as interchain disulfide bonds.

3.1.1. The β_2 subunit of E. coli tryptophan synthase

Chemically unfolded β_2 was shown to refold efficiently under standard renaturation conditions (room temperature, pH 7.8, no additive) only at fairly low protein concentrations [10]. Using the same overall strategy as that previously described for hen lysozyme and β-D-galactosidase [9], the effect of various concentrations of SB256 on the enzymatic activity recovered after renaturation was investigated. At 0.5 mg ml^{-1} of β protein, 20 °C and in standard buffer, the activity recovered was only 7% in the absence of additive, while it dramatically increased with the concentration of SB256 to reach 86% in the presence of 1.9 M SB256 [19]. Furthermore, it was verified that the renatured protein thus obtained remained soluble after removal of the NDSB and was actually native. The protein was dialyzed extensively against NDSB-free buffer and subjected to centrifugation to remove the faint precipitate that appeared in the dialysate. All the enzymatic activity present before dialysis was recovered, indicating that the aggregates in the precipitate contained only inactive molecules. Moreover, the absorption spectrum of the dialyzed protein showed no diffusion, indicating the absence of detectable aggregates. Finally, the specific activity of the dialyzed protein was identical to that of native β_2 which ascertained that the reactivated protein was indeed native and dimeric as authentic β_2 [15]. Thus, as previously observed for hen lysozyme and galactosidase, SB256 appeared to strongly facilitate the folding of β_2.

3.1.2. Bovine serum albumin

Serum albumin is a protein of major pharmacological interest and has been among the first human proteins to be expressed in bacteria [12]. The protein was, however, produced as inclusion bodies and proper folding, which involves correct pairing of 34 cysteinyl side chains into 17 ‘native’ disulfide bonds, turned out to be a difficult process. It could be achieved by dilution only at very low protein concentration [12] or at high protein concentration but only by slow dialysis [1]. Reduced bovine serum albumin (BSA) therefore appeared as a good model system to test SB256 as a folding

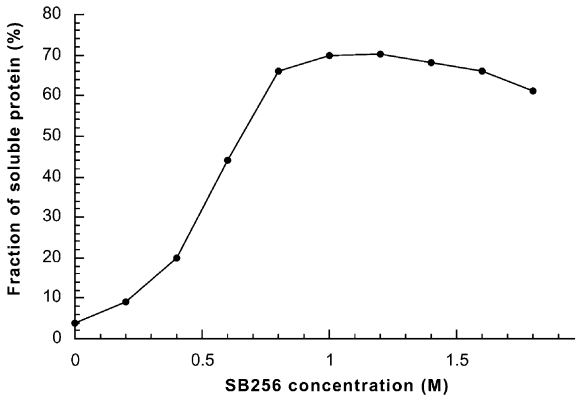


Fig. 1. SB256 concentration dependence of the renaturation yield of BSA. BSA was unfolded/reduced, then renatured/oxidized at a protein concentration of 1 mg ml^{-1} and in the presence of the SB256 concentration indicated in abscissa as described in Section 2. After centrifugation to eliminate insoluble aggregates, the BSA concentration in the supernatent was determined and plotted, in percent of the total BSA submitted to renaturation, as a function of the SB256 concentration.

helper. Because BSA is devoid of catalytic activity, we chose to monitor the efficiency of SB256 by its ability to prevent aggregation, i.e. to help in obtaining soluble BSA molecules at the end of the renaturation/oxidation process. Fig. 1 shows the fraction (as compared to the total denatured BSA submitted to renaturation) of soluble protein as a function of the SB256 concentration present in the renaturation mixture when renaturation was conducted at 1 mg ml^{-1}. A similar curve (not shown) was obtained at 5 mg ml^{-1}. The optimal yield was obtained at 1 M SB256 in both cases, and in both cases SB256 strongly increased the amount of soluble protein recovered. From 4% in the absence of additive, the recovery increased to 73% in the presence of 1 M SB256 when then renaturation was performed at 1 mg ml^{-1} of BSA. Similar results but with lower yields (2% in the absence of additives; 65% at 1 M SB256) were obtained at 5 mg ml^{-1} of BSA during the renaturation. Thus, 1 M SB256 increased the yield in soluble BSA by a factor 18 at 1 mg ml^{-1} and 32 at 5 mg ml^{-1}. That the yield in soluble BSA was significantly lower at 5 than at 1 mg ml^{-1} is likely due to the higher propensity, common to all proteins, to form aggregates at higher concentrations. It should be noted, however, that the dilution procedure we used to initiate renaturation/oxidation resulted in slightly different redox-conditions in the two sets of experiments and may therefore bias a quantitative comparison.

It is noteworthy that, when the renatured/oxidized protein was subjected to extensive dialysis to remove any residual NDSB and centrifuged again to remove aggregates, 95% of the protein present before dialysis was recovered in the supernatent. This indicates that the fraction of soluble renatured/oxidized BSA that was obtained in the presence of SB256 also remained essentially soluble even in the absence of residual NDSB. To better characterize the state of the 'renatured' molecules, the soluble proteins were subjected to PAGE under non-denaturing, non-reducing conditions (Fig. 2). While some low molecular weight aggregates could be detected, most of the protein was recovered as monomers. The majority of the monomers migrated as native BSA, suggesting that they were correctly folded and that the native pattern of disulfides had been established. However, a smear behind the major band indicated the presence of some monomeric misfolded molecules probably containing mispaired disulfides. Most dimeric molecules present in the renaturation mixture migrated more slowly than in the native control. This suggests that they were formed of misfolded monomers, presumably cross-linked by disulfide bonds between cysteines normally involved in intra-chain disulfides as opposed to the dimers of native BSA which are cross-linked by an interchain disulfide between two cysteines that are reduced in native monomeric BSA. Gel filtration of the renatured mixture on a Superdex 75 column also showed essentially the same two peaks as the native BSA, the major one corresponding to the monomeric protein, and the minor one to BSA dimers. The relative amount of the minor peak was slightly larger for the renatured protein (18%) than for the native protein (11%). No further attempt was made to characterize these molecules, or to optimize the renaturation/oxidation procedure, since the results we obtained clearly indicated that SB256 was a powerful

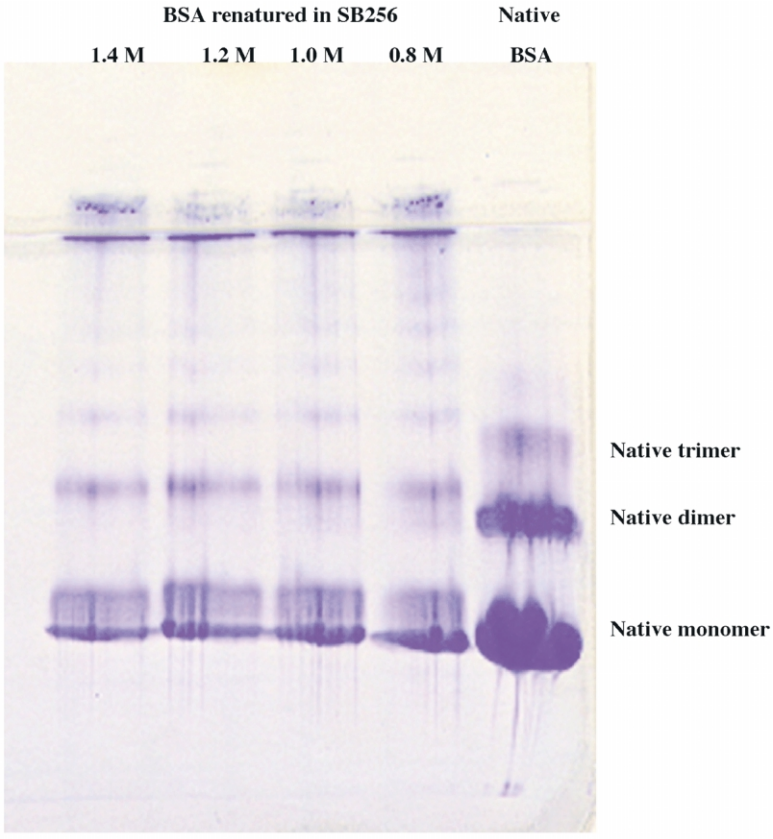


Fig. 2. Electrophoretic migration of refolded/oxidized BSA. Samples of BSA refolded/oxidized at 5 mg ml^{-1} in the presence of various concentrations of SB256 (from left to right: 1.4, 1.2, 1.0, 0.8 M, respectively) were dialyzed against renaturation buffer without SB256. Of each dialysate 10-μl samples were subjected to non-denaturing polyacrylamide gel electrophoresis as described in Section 2. The gels were stained with Coomassie blue. As a control, the right lane contained native BSA.

aggregation inhibitor for BSA even at concentrations in the range of 1–5 mg ml^{-1}.

3.1.3. Anti-tryptophan synthase monoclonal antibodies

Monoclonal antibodies are molecules of considerable interest for diagnostic and therapy purposes and have been the subject of intense engineering efforts. They are, however, extremely difficult to recover in usable amounts in a functional state, particularly when produced in bacteria. Several more or less efficient procedures have been reported for the production of native, functional antibody fragments (Fab, Fv, single chain Fv). Yet, several important applications require that the complete antibody rather than its antigen-binding region be used. Hence our interest in attempts to renature a complete antibody from its unfolded, reduced fragments. For our studies, a monoclonal antibody (mAb 164) that binds, with high affinity, the β_2 subunit of *E. coli* tryptophan synthase was chosen [4,6]. The ability of the renatured protein to bind to immobilized β_2 in an ELISA test was used as a criterion for proper folding. The antibody (an IgG) was first unfolded and reduced in the presence of 6 M GuHCl and 30 mM DTT and renaturation was initiated by a 20-fold dilution into 100 mM Tris–HCl (pH 7.8) buffer supplemented with 2 mM EDTA, 1 mM oxidized DTT (note that the residual reduced DTT concentration

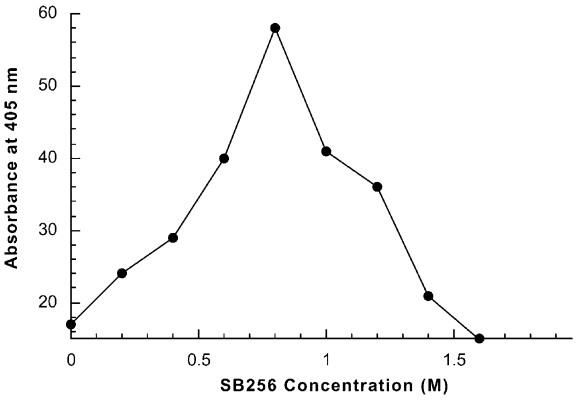


Fig. 3. SB256 concentration dependance of the renaturation yield of mAb164. Samples of mAb164 were refolded/oxidized at a concentration of 50 μg ml^{-1} in the presence of various concentrations of SB256, and then assayed by ELISA as indicated in Section 2. The absorbance measured in the ELISA (proportional to the concentration of active antibody) is shown as a function of the SB256 concentration present in the renaturation/oxidation mixture.

was 1.5 mM) and the desired SB256 concentration. The antibody concentration during refolding was 0.05 mg ml^{-1}. After overnight incubation at 20 °C, the antibody activity was analyzed by ELISA and plotted as a function of the SB256 concentration. Fig. 3 shows a sharp dependence of the yield on the SB256 concentration, with a maximum at 0.8 M sulfobetaine. The amount of binding activity recovered under these conditions was approximately 10% of that measured for native mAb164 at the same total protein concentration, indicating that 10% of active antibody had been recovered. In the absence of SB256, no trace of active antibody could be detected. Again, no effort was made to optimize the renaturation conditions.

Because of the sharp dependence of the yield on SB256 concentration, it was feared that the nearly 'singular point' observed at 0.8 M NDSB might result from an artifact like microaggregates, or non-specific 'sticking' to the plastic. Several controls ruled out this possibility. First, the experiment was repeated three times with similar results. Second, the extend of binding depicted in Fig. 3 was shown to be specific at all SB256 concentrations investigated since no binding was detected either in non-coated wells, or in wells coated with a non-specific protein, or in wells coated with β_2 either in the absence of mAb164 or in the presence of a non-specific mAb. This ascertains that the ELISA test used revealed a specific interaction between the antibody and the antigen. This does not, however, constitute a final proof that native mAb164 molecules were reconstituted. Indeed, correct folding of just one Fv region of the molecule might suffice to create such specific interactions. This result clearly indicates that SB256 acts as a potent helper in the in vitro renaturation/oxidation of mAb164. It should be pointed out, however, that when the optimal renaturation conditions (0.8 M SB256) were used to renature/oxidize an other mouse monoclonal antibody specific for β_2, no binding activity was recovered, which indicated that each individual protein needs specific folding conditions.

Together with results reported previously [9], our observations indicate that SB256 at the appropriate concentrations improves the yield in active (or soluble) protein from 2 to 35% for lysozyme at 1.1 mg ml^{-1}, from 1 to 18% for galactosidase at 0.13 mg ml^{-1}, from 7 to 86% for β_2 at 0.5 mg ml^{-1}, from 2 to 66% for BSA at 5 mg ml^{-1} and from 0 to 10% for the monoclonal antibody mAb 164 at 0.05 mg ml^{-1}. SB256, a member of the non-detergent sulfobetaine family, thus appears as a potent 'universal' folding helper for proteins.

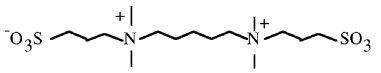
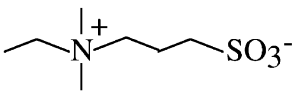
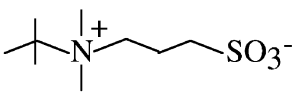
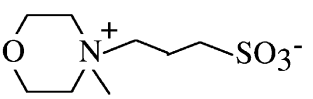
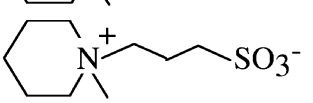
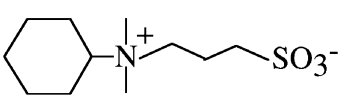
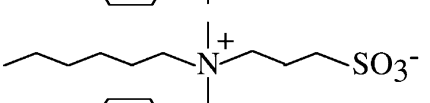
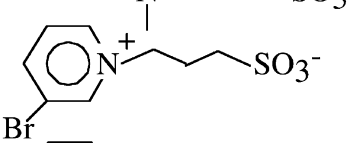
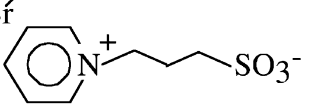
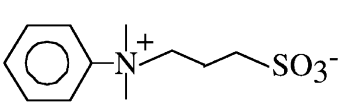
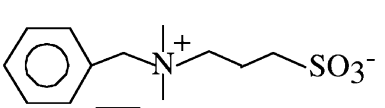
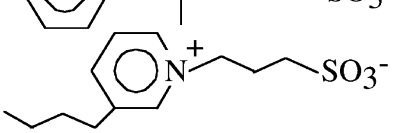
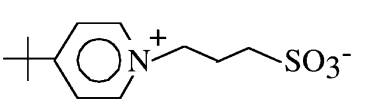
3.2. Effect of a series of NDSBs on the renaturation of lysozyme and β_2

Distinct NDSBs were reported to have widely different effects on protein renaturation [9]. With the aim of understanding the relations between the molecular structure of NDSBs and their efficiency as folding helpers, a variety of NDSB molecules with distinct 'side chains' grafted onto their quaternary amine were investigated. Two proteins were used for this study: hen lysozyme and the β_2 subunit of *E. coli* tryptophan synthase. For each NDSB, unfolded-reduced lysozyme and unfolded β_2 were diluted at the desired concentration in the renaturation buffer in the presence of varying concentrations of NDSB ranging from 0 to 2 M. After incubation, the enzyme was diluted

Table 1

Effect of a series of NDSBs on the renaturation of hen lysozyme and E.coli tryptophan synthase β2 subunit

Hen lysozyme or β2 were denatured, renatured (at 0.5 and 1 mg/ml for β2 and HEWL respectively) at various NDSB concentrations and assayed for enzymatic activity as described in Materials and Methods. The enzymatic activity was corrected for the inhibitory effect of the residual NDSB present in the assay mixture and, for each NDSB, was plotted as a function the NDSB concentration in the renaturation buffer. The table indicates, for each protein and each NDSB, the optimal yield (in % as compared to the inital activity submitted to the denaturation/renaturation cycle), the optimal NDSB concentration (in parenthesis) and the shape of the NDSB concentration dependence curve. The yields in the absence of NDSB were 3 % for lysozyme and 7 % for β2 respectively. ND: not determined.

	Renaturation yield		Curve shape		
	HEWL	β2	HEWL	β2	
416	12 (.4M)	<7	peak	decrease	
195	22 (1M)	<7	increase	decrease	
222t	40 (1.8M)	17 (.8M)	increase	peak	
223	46 (1.4M)	10 (0.6M)	peak	peak	
221	51 (.8M)	34 (1M)	plateau	peak	
249	51 (1M)	77 (1M)	plateau	plateau	
251	51 (1M)	91 (1M)	peak	peak	
253-Br	52 (.6M)	ND	plateau	ND	
201	54 (1M)	30 (1.2M)	plateau	plateau	
244	58 (1M)	45 (0.8M)	peak	plateau	
256	58 (0.6M)	86 (1.8M)	plateau	increase	
256-3N	50 (0.6M)	97 (.4M)	plateau	plateau	
256-4T	60 (0.6M)	100 (1M)	plateau	plateau	

in the assay mixture and the activity was measured. Native enzyme at the same protein concentration was assayed under exactly the same conditions (i.e. in assay buffer supplemented with NDSB at the same residual concentration) to take into account a possible effect of the residual NDSB on the assay. When the yield in active enzyme, expressed as the ratio of the activities of renatured over native enzymes, was plotted as a function of the NDSB concentration, curves of different shapes were obtained. In most cases, a bell-shaped curve, or an ascending curve followed by a plateau were obtained. Table 1 indicates the renaturation yield obtained, for each NDSB, at its optimal concentration. The values obtained for each NDSB and each protein lead one to several conclusions. All the NDSBs tested significantly improve the yield in active lysozyme. This is not true for β_2 for which both SB416 and SB195 exhibit an inhibitory effect, and SB223 hardly shows any activity enhancement. This indicates some specificity in the mode of action of NDSBs. The same conclusion arises by comparing the effects of SB221 and SB251 on lysozyme and β_2. While both NDSBs have the same effect on lysozyme, the yield in β_2 is much higher with SB251 (91%) than with SB221 (34%). More generally, from the range of variations of the optimal yield with the two enzymes, β_2 appears much more sensitive than lysozyme to the nature of the NDSB used.

Using the observed optimal yield in active β_2 as a criterion, one could attempt to identify the structural features of the NDSBs which determine their efficiency as folding helpers. The various NDSBs tested differed in composition and properties of the extension grafted onto the quaternary ammonium of the sulfobetaine motif. For the extension to confer the corresponding NDSB a good folding helper efficiency, it should be a large, hydrophobic motif. Indeed, all active NDSBs had hydrophobic extensions containing at least six carbon atoms. The precise geometry of the extension did not seem to be of crucial importance, since similar effects were observed with linear and aromatic as well as saturated cyclic extensions. An important feature of the most efficient cyclic NDSBs was that the quaternary ammonium was out of the cycle, as evidenced from the comparison of SB221 (34% yield in β_2) with SB249 (77%). Placing the quaternary ammonium out of the cycle (as in SB244) had a much smaller effect in the case of an aromatic cycle. Thus, the optimal yield in β_2 with SB244 (45%) was only slightly higher than that with SB201 (30%). However, introducing one carbon atom between the aromatic cycle and the quaternary ammonium as in SB256 greatly improved the efficiency (86%). This indicated that adding an aliphatic moiety to the cyclic extension of the NDSB improved its efficiency.

3.3. Design and synthesis of optimized NDSBs

Based on the structural features discussed above, we inferred that adding an aliphatic moiety on a cyclic extension, remote from the quaternary ammonium (i.e. in a *para* or *meta*), might enhance the folding helper efficiency of the NDSB. We chose to use SB201 as a reference. Indeed, because its helper efficiency is modest (34% with β_2) an enhancement of the efficiency seemed easier to detect than with a molecule such as SB256 (86% in β_2). Therefore, two new molecules were synthesized, one with a butyl moiety added in *meta*, the other with a trimethyl-methyl-moiety added in *para*. These two molecules, named 256/3N and 256/4T, respectively, were tested on lysozyme and β_2. The results are indicated in the two last lines of table 1. They demonstrate that adding the aliphatic moiety onto the aromatic cycle indeed strongly enhanced the helper efficiency of the corresponding NDSBs. In particular SN2564T appeared as the most efficient folding helper of the series and resulted in a 100% renaturation of β_2, under conditions where the recovery of native protein was only 7% in the absence of NDSB.

4. Conclusion

The studies reported here bring additional information on the use of NDSBs as folding helpers. They confirm that NDSBs help folding in vitro a variety of proteins of highly diverse origins, polypeptide chain lengths, oligomeric states and degrees of internal cross-linking by SS bonds. They show that each NDSB behaves differently with respect to different proteins. Together with

results from independent studies on other proteins [2,3,9,16], our observations indicate that, in difficult cases, it is important to test several NDSBs with different hydrophobicities, typically SB201, SB256 and SB256-4t.

They indicate that a large hydrophobic moiety must be grafted onto the quaternary ammonium of the sulfobetaine motive to enhance the efficiency in helping protein folding.

Finally, they led to the rational synthesis of a new NDSB which, as predicted, turned out to be a very potent folding helper for the two proteins investigated in this study.

NDSBs thus appear as a potentially useful tool to improve folding procedures used in research or production. It should be, however, emphasized that, like any other folding adducts thus far described, a thorough search must be made for each NDSB and each protein to find the optimal conditions best adapted for in vitro renaturation of that protein.

5. Nomenclature

BSA: bovine serum albumin
DTT: dithiothreitol
ELISA: enzyme-linked immunosorbent assay
EDTA: ethylene-diamine-tetraacetic-acid sodium salt
GuHCl: guanidinium chloride
HEWL: hen egg white lysozyme
mAb164: a mouse monoclonal antibody directed against the β_2 subunit of *E. coli* tryptophan synthase
NDSB: non-detergent sulfobetaines
PAGE: polyacrylamide gel electrophoresis
β_2: the β_2 subunit of *E. coli* tryptophan synthase

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