Co-refolding denatured-reduced hen egg white lysozyme with acidic and basic proteins

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Abstract Refolding of denatured-reduced lysozyme and the effect of co-refolding it with other proteins such as RNase A, bovine serum albumin, histone, myelin basic protein, alcohol dehydrogenase and DNase I on the renaturation yield and the aggregation of lysozyme have been studied. Basic proteins consistently increase the renaturation yield of the basic protein lysozyme (10–20% more than in their absence) with little or no aggregation. On the other hand, co-refolding of lysozyme with acidic proteins leads to aggregation and a significant decrease in renaturation yields. Our results show that hetero-interchain interactions (non-specific interactions) occur when the basic protein lysozyme is refolded together with acidic proteins such as bovine serum albumin, alcohol dehydrogenase or DNase I. Our results also suggest that the net charge on proteins plays a significant role in such non-specific aggregation. These results should prove useful in understanding the hetero-interchain interactions between folding polypeptide chains.

Key words: Lysozyme; Refolding; Acidic protein; Basic protein; Inter-chain interaction

1. Introduction

Protein folding studies in vitro have been shown to be important not only to get insight into the fundamental aspects which govern the emergence of functional, three-dimensional structures of proteins from the strings of amino acids [1] but also in biotechnological applications. Two types of interactions determine the folding of proteins to their native state: unimolecular intrachain interactions and multimolecular interchain interactions. Multimolecular interchain interactions usually predominate as the concentration of the protein increases – leading to aggregation and inactivation of the protein [2–8]. Protein folding in vivo occurs in an environment of high protein concentration – the concentration of nascent polypeptide chains on the ribosomes is calculated to be approximately 50 μM [9,10]. Most of the refolding studies are carried out on single proteins and at low concentrations. There are a few studies on the refolding of a single protein at high concentrations where homo-interchain interactions play a major role [2–8]. In this context, it would be interesting to investigate the refolding of mixtures of proteins.

Hen egg white lysozyme has been used as a model protein for refolding studies. Refolding of this enzyme results in varying renaturation yields depending on the conditions employed [2,8,11–13]. In order to understand whether interchain interactions between different folding polypeptide chains affect the outcome of refolding, we have investigated the refolding of denatured-reduced hen egg white lysozyme and the effect of co-refolding it with some basic and acidic proteins such as RNase A, bovine serum albumin (BSA), histone H3, myelin basic protein (MBP) and alcohol dehydrogenase (ADH) on its renaturation process. Such studies would not only mimic in vivo conditions but also provide information on hetero-interchain interactions on the refolding of proteins.

2. Materials and methods

2.1. Materials

RNase A, lysozyme, BSA, ADH, MBP, histone H3, DNase I, dithiothreitol (DTT) and DL-cystine hydrochloride (referred to as cystine) were purchased from Sigma Chemical Company, USA. The purity of these proteins was assessed by SDS-PAGE. BSA was further purified on a Sephacryl S-200 (high resolution) column. Lysozyme was further purified using BioRex-70 column chromatography as described by Saxena and Wetlaufer [11]. The purity of DTT and cystine was analysed by thin layer chromatography on a silica gel matrix; the chromatogram was developed with a solvent mixture of 1-butanol: acetic acid:water (4:2:2 by volume) and visualised using iodine. They were found to be homogeneous.

2.2. Preparation of denatured-reduced proteins

Lysozyme (at 12.5 mg/ml) and other proteins such as BSA, ADH, RNase A, histone H3, MBP and DNase I (at 18 mg/ml) were denatured separately in 50 mM Tris acetate buffer (pH 8.1) containing 6.5 M GdmCl and incubating at 25°C for 1 h. The mixtures of denatured-reduced stocks of proteins (at different weight ratios) were prepared by mixing the required amounts of the above individual denatured stocks in 6.5 M GdmCl and incubating the mixture with 60 mM DTT for approximately 16 h. A denatured-reduced stock of lysozyme alone was also prepared similarly. A stock of DTT (approximately 1 M) in water was prepared and the actual concentration was estimated using 5,5′-dithionitrobenzoic acid [14]. This stock was used for reduction of protein disulphide bonds.

2.3. Refolding experiments

Refolding experiments were performed by a 25-fold dilution of the denatured-reduced stocks of lysozyme alone or of mixtures of proteins in 100 mM Tris acetate buffer (pH 8.1) containing 3.2 mM cystine (refolding buffer). The refolded samples were incubated at room temperature (25°C) for 4 h and lysozyme activity and protein aggregation were measured. In another set of experiments denatured-reduced lysozyme alone was refolded, as mentioned above, in the refolding buffer containing various concentrations of native proteins. The final concentrations of GdmCl and DTT in the refolding buffer after dilution were 0.26 M and 2.4 mM respectively to maintain the same conditions for the refolding of lysozyme either alone or in the mixture. The final lysozyme concentration in the refolding experiments was 0.15 mg/ml. The final concentrations of other proteins were varied from 0.05 mg/ml to 0.3 mg/ml. The extent of aggregation of proteins was measured as turbidity of the refolded samples. Turbidity was measured as optical density at 450 nm using a Hitachi U-2000 UV-Vis absorption spectrophotometer.

2.4. Enzyme assay

Lysozyme activity was determined at 25°C essentially as described by Fischer et al. [13]. The rate of enzymatic lysis of *Micrococcus*

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lysodeikticus cells, suspended in 0.1 M phosphate buffer (pH 6.3), was obtained by measuring the decrease in turbidity of the cell suspension at 450 nm as a function of time using a Hitachi U-2000 UV-Vis spectrophotometer. The percentage renaturation yield in the refolding (or unfolding) studies was calculated with respect to the activity of the native enzyme.

2.5. SDS-PAGE of precipitate obtained on refolding of mixture of proteins

The precipitate obtained on refolding of lysozyme and BSA (1:2) was collected by centrifuging the refolded sample at 5000×*g*. The precipitate was washed three times with the refolding buffer and boiled with SDS-PAGE sample buffer for 3 min. SDS-PAGE of this sample was performed on a 12.5% precast polyacrylamide gel using a Phast-gel electrophoresis system. The protein bands were visualised by Coomassie blue staining.

3. Results and discussion

We have studied refolding of denatured-reduced lysozyme and the effect of co-refolding this protein with some acidic and basic proteins on its renaturation yield and aggregation. Fig. 1 shows the aggregation upon refolding denatured-reduced lysozyme at various concentrations. Aggregation increases as the concentration of the refolding protein increases (Fig. 1A) while the renaturation yield decreases (Fig. 1B). At 0.15 mg/ml lysozyme, we obtained about 70% renaturation yield with negligible turbidity. We chose this concentration of lysozyme for the study of co-refolding with other proteins such as RNase A and BSA. Fig. 1A also shows that the aggregation of RNase A and BSA is significantly less upon refolding at different concentrations (0.05–0.3 mg/ml). Since

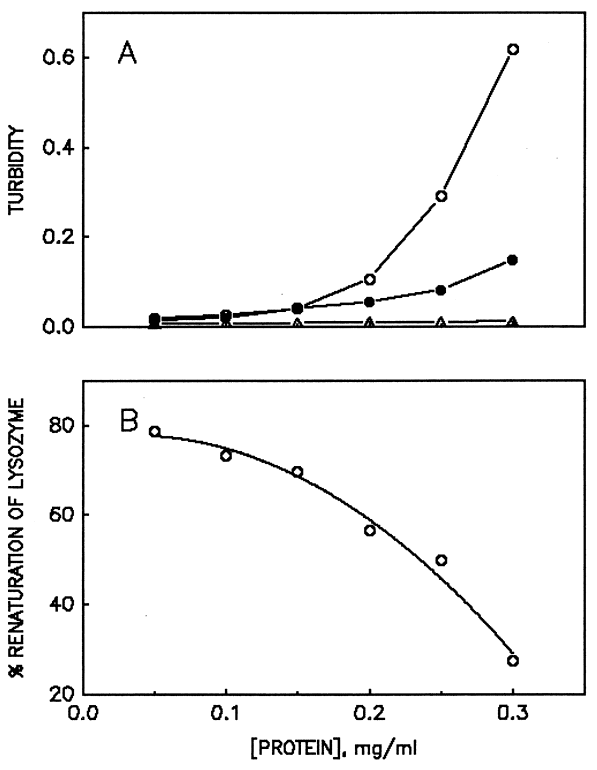

Fig. 1. Refolding of denatured-reduced lysozyme (○), BSA (●) and RNase A (△) at different concentrations. A: Turbidity was measured as optical density at 450 nm. B: The % renaturation yield of lysozyme is the recovery of the activity of lysozyme related to the activity of native enzyme.

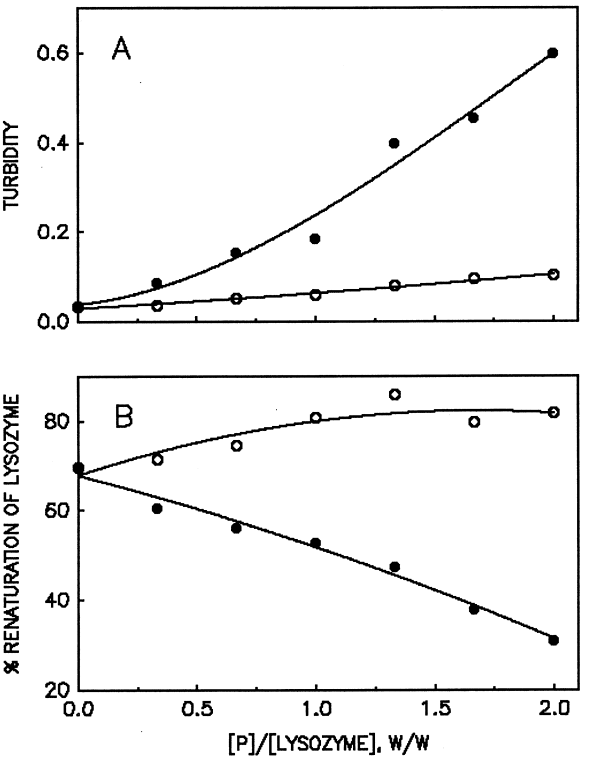

Fig. 2. Co-refolding of denatured-reduced lysozyme with RNase A (○) and BSA (●). A: Turbidity was measured as optical density at 450 nm. B: The % renaturation yield of lysozyme is the recovery of activity of lysozyme related to the activity of the native enzyme. [P] = [BSA] or [RNase A].

our interest was to study the effect of co-refolding of selected proteins on the refolding and aggregation of lysozyme, we did not attempt to characterise the co-refolded proteins.

Fig. 2 shows the aggregation and the renaturation yield of lysozyme when co-refolded with RNase A or BSA. We consistently observed a 10–20% increase in renaturation yield of lysozyme when it was co-refolded with RNase A. While the renaturation yield of lysozyme dropped from 70% to 19% when refolded alone at 0.15 and 0.45 mg/ml respectively (Fig. 1), the renaturation yield of lysozyme (at 0.15 mg/ml) co-refolded with RNase A at a total protein concentration of 0.45 mg/ml was approximately 80%. On the other hand, upon co-refolding lysozyme with varying concentrations of BSA, there was an increase in the measured turbidity of the solution, with a concomitant decrease in the renaturation yield (30% at total protein concentration of 0.45 mg/ml; Fig. 2). We have analysed on SDS-PAGE the precipitate obtained upon co-refolding lysozyme with BSA (Fig. 3). It is evident from the figure that the precipitate contains both polypeptide chains.

Thus, RNase A and BSA exhibit opposite effects on the renaturation of lysozyme. The net charge on lysozyme and RNase A is positive while the net charge on BSA is negative. We therefore investigated whether the net charge on proteins plays a role in the interchain interactions upon co-refolding. Fig. 4 shows that upon co-refolding with ADH, another acidic protein, the renaturation yield of lysozyme decreases and the measured turbidity increases, as in the case of BSA. Similar results were obtained upon co-refolding the acidic protein DNase I with lysozyme. Native ADH, when present in the

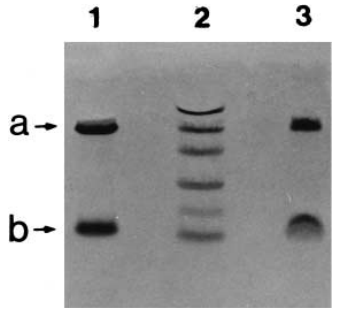

Fig. 3. SDS-PAGE of the precipitate obtained upon co-refolding of denatured-reduced lysozyme with BSA. Lanes: 1, mixture (1:1 w/w) of lysozyme and BSA; 2, low molecular weight markers; 3, the precipitate. SDS-PAGE was performed on a 12.5% polyacrylamide gel.

refolding buffer, decreases the renaturation yield and increases the measured turbidity of lysozyme to a significant extent (Fig. 4), while the presence of other native proteins investigated in this study affects neither the renaturation yield nor the aggregation of lysozyme (data not shown). Fig. 4 also shows the effect of co-refolding lysozyme with basic proteins such as histone H3 and MBP on the renaturation of lysozyme. As seen from Fig. 4 all the basic proteins investigated consistently increased the renaturation yield of the basic protein lysozyme by 10–20%. Interestingly, co-refolding two acidic proteins, BSA and ADH at 0.15 mg/ml each, does not result in significant aggregation (turbidity value of 0.056). When a mixture of lysozyme, RNase A and histone H3 (at 0.15 mg/ml each) is co-refolded, lysozyme refolds without any aggregation to give a high renaturation yield of 89%, even though the total protein concentration is 0.45 mg/ml. Thus, our results show that co-refolding of similarly charged proteins does not lead to significant aggregation while co-refolding of oppositely charged proteins results in aggregation, reducing the folding yields (under the conditions where refolding of individual proteins does not result in aggregation). These results rule out the possibility of a 'molecular crowding effect' in the aggregation observed during the refolding of mixtures of proteins as a general phenomenon, because only the co-refolding of oppositely charged proteins, but not of similarly charged proteins, results in aggregation. When lysozyme (0.15 mg/ml) is refolded together with the mixtures of all the acidic and basic proteins at a total protein concentration of 0.45 mg/ml, the renaturation yield of lysozyme is comparable to when it is refolded alone (approximately 70%). This suggests that when a mixture of acidic and basic proteins is refolded, competitive interactions between folding hetero-polypeptides mutually modulate the final outcome of the refolding reaction.

Understanding the mechanism of protein aggregation is important, because (i) several diseases are caused by either mutations or environmental changes that divert proteins from their normal folding pathway leading to aggregation of proteins in vivo [15–17] and (ii) biotechnological applications to produce several proteins often lead to aggregation of incompletely folded proteins, inclusion bodies [18,19]. The mechanism of protein aggregation has been thought to involve two possible interchain interactions: (i) non-specific interactions between hetero-polypeptide chains (coagulation) [2,5] and (ii) specific interactions through complementary surfaces between homo-polypeptide chains [4,20–22].

Specific and non-specific interactions could occur between folding polypeptide chains and the magnitude of the individual interactions could determine the overall interpolypeptide chain interactions. Non-specific hydrophobic interactions between hetero-polypeptide chains may often be weak and transient compared to the specific interactions through complementary surfaces. Opposite charges on the hetero-polypeptide chains might increase the proximity (or the chance of collision of two hetero-polypeptide chains) and tend to stabilise the non-specific hydrophobic interactions and hence promote the hetero-interchain association. From our results, it is evident that such non-specific interactions do occur when the basic protein lysozyme is co-refolded with acidic proteins. Thus, our results provide a correlation between the observed non-specific interactions and net charge on proteins. We therefore hypothesise that non-specific hetero-interchain interactions do occur in some cases and net charge on proteins plays a significant role in such non-specific aggregation. On the other hand, it is possible that charge repulsion between two similarly charged proteins might tend to reduce the intermolecular interactions, leading to productive folding of lysozyme. This may be reason for the observed increase in the renaturation yield of lysozyme when it is co-refolded with other basic proteins. This raises the question: why does lysozyme aggregate at high concentrations in spite of all molecules having the same net charge? The aggregation of lysozyme might involve a strong specific hydrophobic association of complementary surfaces exposed during refolding, which may overcome the repulsive charge interactions. Brems and coworkers [4,21] have demonstrated that specific interactions between homo-polypeptide chains through hydrophobic complementary surfaces are a predominant force involved in protein aggregation. Goldberg et al. [2] observed that the presence of denatured hen egg lysozyme reduced the renaturation yield of turkey lysozyme with the formation of hybrid aggregates, though the two enzymes have a similar net charge. Sequence analysis shows that the two enzymes are highly homologous and share more than 95% sequence identity. Highly homologous proteins may have complementary surfaces which can interact.

London et al. [22] observed that the renaturation yield of tryptophanase remained unaffected upon co-refolding with BSA or *Escherichia coli* cell lysate and concluded that foreign proteins do not interfere with the refolding of tryptophanase. A recent study of Speed et al. [20] shows that P22 tailspike and P22 coat proteins do not co-aggregate with each other but only self-aggregate on co-refolding them, indicating that aggregation occurs by specific interactions of certain conforma-

Table 1
Isoelectric points (p*I*) of proteins referred to in the study

Protein	p*I*	Reference
Lysozyme	10.5–11.0	[26]
RNase A	9.3	[27]
Histone H3	11.0	[28]
MBP	11.9	*
DNase I	4.7–5.0	[29]
BSA	4.7–5.3	[30]
ADH	5.4	[31]
Tryptophanase	5.8	*
P22 endorahmnosidase	5.1	*
P22 coat protein	4.7	*

*p*I* computed based on sequence using PC-Gene (IntelliGenetics Inc., using the program Charg Pro).

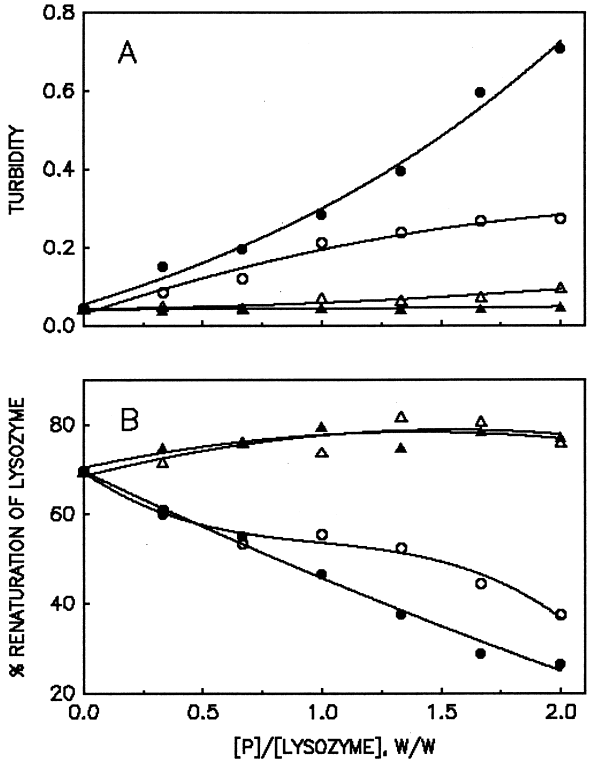

Fig. 4. Co-refolding of denatured-reduced lysozyme with alcohol dehydrogenase (●), histone H3 (△) and MBP (▲). Refolding of denatured-reduced lysozyme in the presence of native alcohol dehydrogenase was also performed (○). A: Turbidity was measured as optical density of the samples at 450 nm. B: The % renaturation yield is the recovery of activity of lysozyme related to the activity of the native enzyme.

tions of their folding intermediates. It is interesting to note that the proteins, tryptophanase and BSA, chosen in the study of London et al. [22] or the P22 tailspike and P22 coat proteins chosen in the study of Speed et al. [20] are similarly charged (acidic; see Table 1). Our present study shows that oppositely charged proteins co-precipitate whereas similarly charged proteins do not co-precipitate (Figs. 2–4). This is probably why they did not observe any non-specific interactions in their study.

In the context of interpolypeptide interactions, it is interesting to note that hydrophobic interactions are the major forces involved in interactions between the native molecular chaperones and the folding substrate proteins [23], preventing the aggregation of the folding proteins. The interaction seems to be non-specific with respect to the substrate proteins and specific with respect to the conformations of the partially folded proteins which are recognised by the chaperone molecules [23]. Electrostatic interactions also appear to play a role in the interaction between folding protein and the chaperonin GroEL [24]; the interaction between the basic protein barnase and the acidic protein GroEL [25] is much stronger than the interaction between the acidic protein α-lactalbumin and GroEL [24]. It would be interesting to know whether interchain interactions between different folding polypeptide chains also play a role in the in vivo protein folding besides the role of molecular chaperones.

We conclude that acidic and basic proteins (non-homologous) have different and opposite effects on the final renaturation yield and aggregation of lysozyme when they are co-refolded with lysozyme. Our results thus suggest that the net charges on proteins mutually modulate the productive folding and non-productive aggregation of the folding polypeptide chains. These results should prove useful in understanding the role of hetero-interchain interactions between folding polypeptide chains.

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