Reactive & Functional Polymers 73 (2013) 1405–1411

Contents lists available at SciVerse ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

Mechanistic studies of protein refolding facilitated by like-charged polymers

Chun-Yan Yang, Xiao-Yan Dong, Yan Sun *

Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form 2 June 2013
Accepted 3 July 2013
Available online 11 July 2013

Keywords:
Protein refolding
Polyelectrolyte
Like-charge
Critical total charge ratio
Critical molecular weight

ABSTRACT

The mechanism of lysozyme refolding facilitated by like-charged polymers was studied using cationic polyelectrolytes of different molecular weights and structures. Lysozyme refolding yield increased with increasing the total charge ratio (R) of the charged polymer to lysozyme in the refolding solution till reaching a plateau at a critical total charge ratio (R_c). The same R_c was observed for different polymers. Similarly, there was a critical minimum polymer molecular weight (M_c) for the facilitated protein refolding, below which the refolding yield decreased. The refolding yield was independent of the charge group structures in polymer. Fluorescence spectroscopy revealed that the polymers had no effect on the protein folding kinetics. Two physical models were proposed to explain the mechanism of the facilitated refolding and the meanings of R_c and M_c. The facilitating effect was attributed to the electrostatic interaction-induced oriented alignment of multiple protein molecules near the polymer chains, which maximizes the electrostatic repulsion between neighboring protein molecules, leading to the inhibition of protein aggregation. The studies provided insight into the mechanism of like-charged polymer-facilitated protein refolding, which would help develop more efficient polymers/particles for facilitated protein refolding applications.

1. Introduction

Although gene recombination technology has made it easy to design and express heterologous proteins in bacteria systems [1–3], it often encounters a difficulty of insoluble aggregate formation of recombinant proteins expressed by these systems [4]. Hence, it is necessary to refold *in vitro* the polypeptides into their native and active conformations. During a refolding process, hydrophobic interaction-induced aggregation between folding intermediates compete with the on-pathway folding, leading to a low refolding yield, especially at high protein concentrations [5–7]. Therefore, inhibition of the aggregation becomes the key strategy for enhancing protein refolding [8–10]. To inhibit the protein aggregation, various folding aids have been employed in refolding systems, including traditional dilution additives such as low concentration of denaturants (e.g., urea and guanidine hydrochloride), arginine, and surfactants [11–14], and some novel functional macromolecules, such as some amphiphilic macromolecules [15–17]. However, most of the additives effective in suppressing protein aggregation also compromise the intramolecular hydrophobic interactions, which are necessary for protein folding [18], leading to a low refolding rate [19].

Our group has found that addition of like-charged resin particles in a refolding solution can greatly enhance protein refolding at high protein concentrations [20]. The effect of solid phase properties of like-charged media on lysozyme refolding was studied subsequently with positively charged agarose gels [21] and mono-sized microspheres [22]. It has been proposed that the electrostatic repulsion between like-charged particles and the protein (folding intermediates) induced an oriented alignment of the folding protein molecules near the charged solid surfaces, which could maximize the electrostatic repulsion between protein molecules, leading to the suppression of protein aggregation [20]. However, the earlier studies have mainly focused on the examination of refolding yields, and the use of solid particles made it difficult in the real-time analysis of protein folding and aggregation behaviors. As a result, some detailed information on the facilitated refolding behavior is still unclear.

In view of this limitation of solid particles, we have herein studied the effects of soluble polyelectrolytes on like-charged protein refolding. The properties of the homogeneous system make it possible in the measurement of the real-time changes of solution fluorescence intensity and turbidity. These measurements can unveil our concerns about the folding and aggregation kinetics in the presence of like-charged polymers (particles). For this purpose, cationic polyelectrolytes of different molecular weights and structures were used to investigate their effects in lysozyme refolding,

* Corresponding author. Tel./fax: +86 22 27404981.
E-mail address: ysun@tju.edu.cn (Y. Sun).

1381-5148/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.reactfunctpolym.2013.07.005

which has an isoelectric point of 11 [23] and is positively charged under the refolding condition (pH 8.5). The research is expected to deepen our understanding of the protein refolding facilitated by like-charged particles/polymers, and to help develop more efficient materials for this facilitated refolding purpose.

2. Experimental

2.1. Materials

Chicken egg white lysozyme and *Micrococcus lysodeikticus* were purchased from Sigma–Aldrich (St. Louis, MO, USA). The polymers used in lysozyme refolding are listed in Table 1. In the table, poly(diallyldimethyl ammonium chloride) (PD), poly(ethyleneimine) (PEI), and poly(L-lysine) (PL) were obtained from Sigma–Aldrich (St. Louis, MO, USA), while poly(vinylamine) (PVAm) was from PolyScience (Niles, IL, USA) and polyethylene glycol (PEG) was a product of Dingguo Biotech (Beijing, China). The abbreviations of the polymers according to their nominal molecular weights are given in Table 1. Glutathione oxidized (GSSG), glutathione reduced (GSH), urea, tris(hydroxymethyl)aminomethane (Tris) and dithiothreitol (DTT) were also from Dingguo Biotech (Beijing, China). Ethylenediaminetetraacetic acid disodium (EDTA) was purchased from Guangfu Fine Chemical Research Institute (Tianjin, China).

2.2. Determination of polymer sizes

The molecular weights of the polymers were measured by size-exclusion chromatography (SEC) with a ZORBOX GF-250 column equipped on the Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). Details about the methods are described in the Supporting information, and the results are listed in Table 1.

2.3. Charge number calculations

The charge concentration of a cationic polyelectrolyte (or lysozyme) in the refolding solutions was calculated from the following equation:

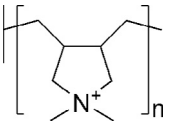
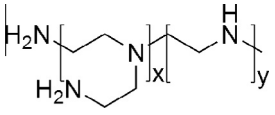
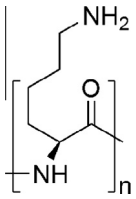
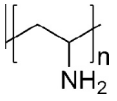
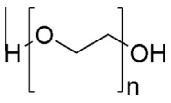
$$N = Z \cdot c / M_w \quad (1)$$

where N is the charge concentration of a polyelectrolyte or lysozyme in mol/L, c the polyelectrolyte or lysozyme concentration in mg/mL, Z the molecular charge number, and M_w the molecular weight in g/mol. The molecular charge numbers of the polyelectrolytes and lysozyme at pH 8.5 were determined by the colloid titration as described previously [24,25]. The molecular charge number of lysozyme at pH 8.5 is determined to be 6.9, and those of the polyelectrolytes are listed in Table 1. The M_w values of polyelectrolytes in Eq. (1) refer to the measured M_w values as listed in Table 1. The total charge ratio of a polyelectrolyte to lysozyme in a refolding solution was calculated from the following equation:

$$R = N_P / N_L \quad (2)$$

where R is the total charge ratio of a polyelectrolyte to lysozyme in a solution, N_P the polyelectrolyte charge concentration, and N_L the lysozyme charge concentration.

Table 1
Molecular weights (M_w), molecular charge numbers (Z), functional groups, and structures of the polymers used in this work.

Polymer	Nominal M_w[a] (kDa)	Measured M_w[b] (kDa)	Z[c]	Functional group	Structure
Poly(diallyldimethyl ammonium chloride)	–	1.8	13.6	$-N^+R_4$	
	100–200 [PD-M]	142	1115		
	400–500 [PD-L]	415	3359		
Poly(ethyleneimine)	1.2 [PEI-S]	1.2	13.2	$-NH_2R_1$, $-NHR_2$, $-NR_3$	
	60 [PEI-L]	62	652		
Poly(L-lysine)	30–70 [PL-S]	45	450	$-NH_2R_1$, $-NHR_2$	
	70–150 [PL-M]	104	1015		
	150–300 [PL-L]	216	2134		
Poly(vinylamine)	340 [PVAm]	318	5324	$-NH_2R_1$	
Polyethylene glycol	20 [PEG]	–	–	R–O–R	

[a] Nominal molecular weights of polymers were provided by the manufacturer. The abbreviations of the polymers are given in brackets according to their nominal molecular weights.
[b] Measured molecular weights were determined by size-exclusion chromatography (SEC) as described in Section 2.2.
[c] The molecular charge numbers at pH 8.5 were determined by the colloid titration as described in Section 2.3.