Biochemical Engineering Journal 46 (2009) 7–11

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

The role of disulfide bond formation in the conformational folding kinetics of denatured/reduced lysozyme

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ARTICLE INFO

Article history:
Received 16 February 2009
Received in revised form 2 April 2009
Accepted 7 April 2009

Keywords:
Bioseparations
Protein
Refolding
Kinetic Parameters
Oxidative folding
Disulfide

ABSTRACT

Oxidative folding is of vital importance for producing therapeutic proteins in bacteria via recombinant DNA technology since disulfide bonds exist in most pharmaceutical proteins. Although oxidative protein folding has been extensively investigated *in vitro*, little is explored concerning the role of disulfide formation to protein conformational folding rate. The effects of oxidized (GSSG)/reduced (GSH) glutathione and pH on the conformational folding kinetics of denatured/reduced lysozyme have been studied herein by fluorescence and circular dichroism. It is found that 83% tryptophan residue burial requires disulfide formation, and increasing GSSG concentration greatly accelerates the tertiary structure formation. The fast phase folding rate constant (k_1) is linearly related to GSSG concentration, indicating the rate-limiting role of mixed-disulfide formation. Moreover, $k_1 = 0.006(\pm 0.001)\,s^{-1}$ is likely to be a critical value for judging the determinant of the slow phase folding rate (k_2), namely, k_2 is controlled by disulfide formation rate only at $k_1 < 0.006\,s^{-1}$. These findings have elucidated the determinants of different folding stages and thus may be beneficial for more efficient control of the oxidative folding of proteins.

1. Introduction

Oxidative protein folding is a composite process in which disulfide bond regeneration and native conformational folding are both achieved. Hence, it is of significance to elucidate the relationship between disulfide regeneration and protein conformational transition for effectively manipulating the process of therapeutic protein production via genetic engineering [1,2]. Besides, oxidative protein folding study is also important for understanding the mechanisms of some important life [3,4] and disease-related processes [5,6] *in vivo*.

The composition of redox buffer is a key parameter for *in vitro* oxidative folding. Currently, oxidized glutathione (GSSG) and reduced glutathione (GSH) are often used for modulating the formation and reshuffling of disulfide bonds. Researchers have made many efforts to find the optimum proportion and concentration of the redox pair [7]. For example, their effects on the activity recovery kinetics [8,9] and on the folding pathway of ribonuclease A [10–12] and bovine pancreatic trypsin inhibitor [13] have been investigated comprehensively. Nevertheless, questions remain regarding how it is related to the conformational transition rate during oxidative folding. Elucidating this question is essential to completely unravel the way disulfide formation works on the oxidative folding of proteins. This work has addressed this question using chicken egg white lysozyme as the model protein.

Chicken egg white lysozyme is a globular protein containing four disulfide bonds. Till now, the oxidative folding of lysozyme has been well studied, including its disulfide folding pathway [14–16] and folding kinetics [17–21]. However, these studies revealed little about what are the rate determinants of different phases. Nor did they indicate the kinetic relationship between disulfide regeneration rate and tertiary structure formation rate during oxidative folding. Hence, we have studied the effects of GSSG and GSH, as well as pH on the conformational folding of denatured and reduced lysozyme.

2. Materials and methods

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), GSH, GSSG, *Micrococcus lysodeikticus* and chicken egg white lysozyme were from Sigma (St. Louis, MO, USA). Urea was obtained from Genview (Houston, TX, USA). EDTA was purchased from Guangfu Fine Chemical Research Institute (Tianjin, China). All the materials were used as received.

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doi:10.1016/j.bej.2009.04.005

2.2. Denaturation and reduction of lysozyme

Lysozyme was dissolved in denaturing buffer (8 M urea, 100 mM Tris–H_2SO_4, 1 mM EDTA and 100 mM DTT, pH 8.5) at a final concentration of 1 mM for fluorescence and 5 mM for circular dichroism (CD). It was completely denatured and reduced by incubation at 40 °C for 3 h.

2.3. Folding kinetic experiments

The folding dynamics studied by intrinsic fluorescence followed the procedure described previously [20]. Fluorescence was monitored in real-time by a PerkinElmer LS 55 fluorescence spectrometer (Beaconsfield, Bucks, UK) with excitation/emission at 280/350 nm and slit width at 2.5/3.0 nm. Folding kinetics studied by near-UV CD were done as described previously [20] using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) with 1 cm cell at 289 nm.

To study the effect of GSSG and GSH concentrations, the reduced/denatured protein was first diluted with the denaturing buffer without DTT. Then, the solution was further diluted 10-fold for fluorescence (four-fold for CD) with refolding buffer (100 mM Tris–H_2SO_4, 1 mM EDTA, predetermined concentrations of GSH and GSSG, pH 8.5). The final solution contained 1 μM lysozyme and 0.8 M urea for fluorescence, but 5 μM lysozyme and 2 M urea for CD. GSH solution was freshly prepared for each experiment. Since the reaction between DTT and GSSG is very fast, the GSH and GSSG concentrations reported were the equilibrium values between GSSG/GSH and DTT calculated from the equilibrium constant at pH 8.7, 25 °C reported by Rothwarf and Scheraga [22].

To study the effect of pH by fluorescence, oxidative renaturations over a pH range from 7.3 to 8.5 were achieved by adjusting the pH of the refolding buffer. GSH and GSSG concentrations were fixed at 1.0 and 0.9 mM, respectively. All other conditions were kept the same as described above.

The refolding buffer was degassed and flushed with nitrogen before use. Folding reactions were all carried out at 25 °C. In all cases, rapid mixing of solutions to initiate refolding was performed manually. The typical dead time in these experiments was about 10 s.

In light of previous findings that the folding kinetics of lysozyme can be well fitted to double exponential function [17,20], all kinetic data were fitted to single or double-exponential function with ORIGIN 7.0 (OriginLab, Northampton, MA, USA). For the data we got, double-exponential function gave better fitting results. The double-exponential function is expressed as

$$y = y_0 + A_1 \exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right) \quad (1)$$

or

$$y = y_0 + A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \quad (2)$$

where t is folding time, y the fluorescence at time t, y_0 the fluorescence intensity at infinite time, A the amplitudes, τ the folding rate time constants, and k the folding rate constants. The subscripts 1 and 2 for the parameters correspond to the first and second kinetic phases, respectively. The kinetic constants were obtained as the averages of two or three repeated experiments.

2.4. Kinetics of intensity average wavelength change

To compare the tryptophan burial of lysozyme during folding in the presence and absence of GSSG, the folding processes were detected by the change in tryptophan fluorescence emission between 300 and 500 nm after excitation at 280 nm. The intensity average wavelength was obtained as described by Royer et al. [23].

2.5. Assay of enzymatic activity

Lysozyme activity was assayed using *M. lysodeikticus* as the substrate as described earlier [24]. In the assay, 1.25 mL of 0.25 mg/mL substrate solution was mixed completely with 0.25 mL protein solution (1 μM) at 25 °C. The decrease in the absorbance of the mixed solution at 450 nm was then recorded. The slope of the absorbance curve till 90 s was used for calculation of the relative activity by comparison with the slope obtained with the native enzyme.

3. Results and discussion

3.1. Disulfide formation is a prerequisite for tryptophan burial

To investigate the effect of disulfide formation on tryptophan burial, we drew on fluorescence intensity average emission wavelength (AEW). Compared with the total intrinsic fluorescence, AEW is a sensitive factor to monitor local tryptophan environment [23]. Likewise, other researchers have used it to characterize the collapsed states in the refolding of non-reduced lysozyme [25].

From the denatured/reduced state to the native state, there was a 5.97-nm change of the AEW (Fig. 1). Only a small change of AEW (1.02 nm) happened during the very fast hydrophobic collapse in the dead time of the experiment. In the monitoring period (70 min), the AEW of the reduced/denatured lysozyme had almost no change in the refolding buffer without GSSG (Fig. 1). The spectrum obtained after the 70-min refolding was almost the same as that acquired at the initiation of the refolding (Fig. 2). It indicates that there was little tryptophan burial in the absence of GSSG. By contrast, the tryptophan burial happened rapidly in the refolding buffer with 1 mM GSSG; within 70 min, the protein almost reached the native conformation (Fig. 2). The time course of the AEW change could be well fitted to a double-exponential curve (Eq. (1)) ($R^2 = 0.985$). The fitting gave time constants of $\tau_1 = 1.26(\pm 0.43)$ min and $\tau_2 = 9.02(\pm 0.66)$ min for the first and second phases, respectively, corresponding to the first two kinetic phases reported by Berg et al. ($\tau_1 = 3.33$ min, $\tau_2 = 16.7$–28.3 min) [20]. Obviously, the time constants we got are smaller than those presented by Berg et al. The discrepancy comes from the different folding conditions. We measured the folding at higher concentration of GSSG (Fig. 4), higher temperature and lower urea concentration; all contribute to the higher folding rates obtained in this work.

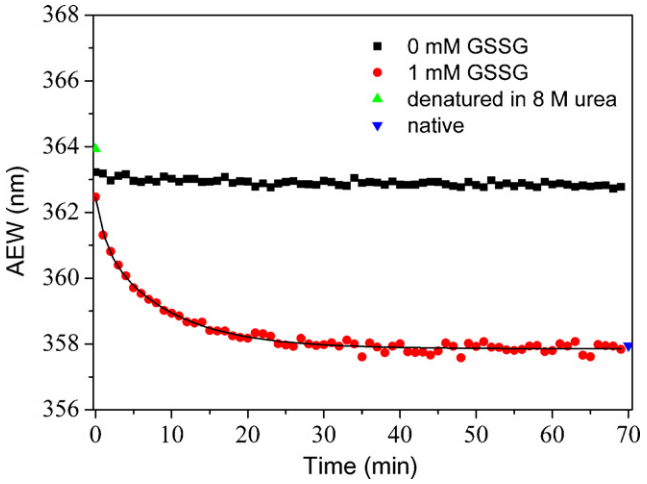

Fig. 1. The time course of average emission wavelength (AEW) change during the oxidative folding of lysozyme with 0 mM (■) and 1 mM (●) GSSG. ([GSH] = 1.0 mM). The AEW value of the denatured reduced lysozyme in 8 M urea is marked as "▲" and the native lysozyme in refolding buffer as "▼".