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Reduced and mutant lysozyme refolding with lipid vesicles. Model study of disulfide impact on equilibria and dynamics

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ABSTRACT

The recovery of secondary structure in disordered, disulfide-reduced hen egg white lysozyme (HEWL) upon interaction with lipid vesicles was studied using circular dichroism (CD), fluorescence and infrared (IR) spectroscopic techniques. Lipid vesicles having negative head groups, such as DMPG, interact with reduced HEWL to induce formation of more helical structure than in native HEWL, but no stable tertiary structure was evident. Changes in tertiary structure, as evidenced by local environment of the tryptophan residues, were monitored by fluorescence. Spectra for oxidized HEWL, reduced HEWL and mutants with no or just one disulfide bond developed variable degrees of increased helicity when added to negatively charged lipid vesicles, mostly depending on packing of tails. When mixed with zwitterionic lipid vesicles, reduced HEWL developed β-sheet structure with no change in helicity, indicating an altered interaction mechanism. Stopped flow CD and fluorescence dynamics, were fit to multi-exponential forms, consistent with refolding to metastable intermediates of increasing helicity for HEWL interacting with lipid vesicles. Formation of an intermediate after rapid interaction of the lipid vesicles and the protein is supported by the correlation of faster steps in CD and fluorescence kinetics, and largely appears driven by electrostatic interaction. In subsequent slower steps, the partially refolded intermediate further alters structure, gaining helicity and modifying tryptophan packing, as driven by hydrophobic interactions.

1. Introduction

Most proteins can spontaneously fold to form a unique compact structure that is biologically active, and the realization of this process in a variety of environments remains a fundamental question in biophysical studies [1]. While extensive insight into the mechanism of protein folding has developed over the last few decades, the field remains active with a variety of ongoing studies, many emphasizing the effects of sequence modification on attaining the global folded minimum [2,3]. In particular, studies of intrinsically disordered proteins (IDP) that have modest or low levels of stable (secondary or tertiary) native structure but fold to a more ordered state on interacting with other proteins, lipids or other perturbants have become a major subdiscipline [4,5]. This can be a particularly important aspect of investigations of protein misfolding that is symptomatic of important biomedical pathologies, for example in amyloid related neurotoxic diseases.

A major aspect of many protein structures is the formation and role of disulfide bonds in stabilizing their active forms [6]. A number of previous studies have addressed this for various proteins. One of the most thoroughly studied protein models for folding studies is hen egg-white lysozyme (HEWL), which has four disulfide bonds. By reduction of these S—S linkages or by mutation of the Cys residues, the role of disulfides in folding of HEWL can be altered [7–22]. HEWL loses detectable secondary and tertiary structure on reduction, becoming structurally equivalent to an IDP. In general, reduction of disulfides leads to a decreased stability relative to the native state structure [8,9,13,19,23–26] and can lead to aggregation and other irreversible misfolded structures [27].

The role of disulfides in protein folding has been viewed in different ways. While they have a stabilizing effect on the low-energy equilibrium structure, some studies indicated the disulfide links form late in the folding process, having little or no influence on the pathway to the global minimum [14,15,28,29]. Experimental and simulation modeling results have supported this for specific small protein systems, but do not address the role of preformed disulfides, which are often present in protein folding studies based on denaturing native proteins and refolding by removal of the denaturant. For these cases, the opposite view of this mechanistic path is relevant, i.e. that the formation of native disulfide bonds can greatly accelerate the folding process by constraining tertiary structure before a compact arrangement is formed [17]. This is certainly true for HEWL, where folding of reduced HEWL

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after oxidation is much slower than for renatured oxidized HEWL [14,16,30]. Intermediate between these mechanistic models are studies showing that selected disulfides can be modified while still allowing the protein to develop native-like secondary structure [21]. Despite a number of studies available, and recent support for the former mechanistic view from calculational results on specific protein models [28], reasonable questions remain on the role of disulfides in determining the folding pathway for a variety of proteins.

Interaction with membranes can alter folding of many protein systems, and of course membrane proteins rarely fold to a native state without the influence of the unique membrane bilayer environment. HEWL is known to be influenced by change in its environment, such as by addition of trifluoroethanol (TFE), which lowers the activity of the protein's aqueous solvation and has sometimes been claimed to model effects of membrane interaction [31–33]. We have shown that HEWL interacts with surfactants and that reduced HEWL becomes substantially helical, even more so than native (oxidized), upon mixing with anionic micellar surfactants, at concentrations above their CMC (critical micelle concentration) [7]. Those surfactants having negative charges are the most effective, yet positive ones can also induce reversion of structure. Micellar interaction is different than lipid bilayer interaction in that various mechanisms are possible, wherein the micelles can stabilize hydrophobic groups or interact with specific parts of the unfolded protein to promote structural change. Lipid bilayers are more restricted from this point of view, provide an interacting surface layer whose charge, structure and organization can be varied and are also of more biological interest.

Most previous studies of protein folding and the basic principles governing the folding process have been carried out using renaturation of proteins that maintain native-like disulfide bonds or other chemical cross-links, although some have explored the rate of reformation of these bonds [14,16,25,30]. Maintenance of these native features reduces the configurational space the protein must explore to fold and templates the folding process, to some extent. However in nature, proteins emerge from the ribosome in the reduced form and then fold through various mechanisms, initially without preformed disulfide bonds. Consequently, to more closely mimic this potential initial aspect of the physiological folding process, in this study we study the refolding of a model system, fully reduced hen egg white lysozyme (HEWL), as well as two Cys → Ala HEWL mutants, one of which has no disulfide bonds (0SS) and another which has only one disulfide bond left (1SS) [34], and compare the results to those of native HEWL with four intact disulfide bonds. Without some initiating environmental perturbation, these structures would remain unfolded, much as do IDPs, so we have studied their spectral changes when mixed with model lipid vesicles with which they can interact and partially refold. Our previous studies reported on induced folding of reduced HEWL when mixed with surfactant micelles [7], and we now extend those studies to HEWL interactions with lipid vesicles which can provide a more relevant model of protein-membrane interactions.

The HEWL mutant data, for 0SS and 1SS, provide control systems that lack all or all but one of the four native HEWL disulfide bonds but are studied under conditions that do not require any reductant [25]. The 1SS mutant C64-C80 used here has its lone disulfide bond left in the β domain, which is the only intra-β-domain disulfide bond in native HEWL. By comparing the refolding process of the 1SS mutant with one intra-β-domain disulfide bond remaining intact, we can study the effects of inter-domain contact via this single disulfide bond and its role in the change to helical structure on binding to lipid vesicles. In this work we use HEWL interaction with lipid vesicles as a model system to study the impact of disulfides on forming organized protein structure in a structure promoting environment. While HEWL is not a membrane protein, its interaction with a membrane-like system yields large changes that can be monitored spectroscopically and give insight into the relative paths for folding with and without disulfides in a membrane-like environment. Its behavior further mimics that of IDPs, but additionally has a reference native structure that can be used as a control for structure formation capability.

2. Experimental

2.1. Materials

Lysozyme from hen egg white (HEWL) was purchased from Sigma (catalog no. L6876), as were the reducing agent 1,4-dithio-DL-threitol (DTT), organic solvents (methanol and chloroform, spectral-grade), and sodium phosphate (analytical grade). The lipids used: dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidycholine (DMPC), dilauroylphosphatidylglycerol (DLPG), dioleoylphosphatidylglycerol (DOPG), and palmitoylphosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The HEWL 0SS and 1SS mutants were prepared in the Schwalbe lab, Universität Frankfurt, as described previously [25,34]. Solution pH was measured with a HANNA HI 98180 pH meter. All the materials were used without further purification.

To prepare reduced lysozyme solution, the protein was dissolved (0.4 mg/mL) in 20 mM pH 4.6 phosphate buffer and incubated with 5 mM DTT for 24 h at 65 °C. The reduction of the sample was quenched by rapid cooling and then storage at 5 °C. This procedure is a modification of a previously published method but required longer incubation times to attain complete reduction, due to the lower pH conditions that were used to enhance solubility of the reduced HEWL form with DTT reductant [35].

2.2. Lipid vesicles preparation

A weighed amount of lipid was dissolved in organic solvents (chloroform or chloroform/methanol mixture) in a glass vial, and the resulting lipid solutions were dried while being rotated under a small stream of dried air to form a relatively uniform thin film on the wall. The required volume of 20 mM pH 4.6 phosphate buffer was added to the vial to resuspend the dried film and form a multilamellar liposome suspension. The suspension was then sonicated (MICROSON XL2000 ultrasonic liquid processor, Qsonica, LLC) with 5–6 W power for a few minutes until the solution became clear. This freshly prepared solution of small unilamellar vesicles (SUV) was then added to the protein solution and mixed well to form a protein-lipid complex with a final 0.2 mg/mL HEWL concentration. The resulting SUV sizes were broadly dispersed but averaged ~50 nm as measured with dynamic light scattering (DLS).

2.3. Circular dichroism measurements

CD spectra were measured from 185 nm to 250 nm with a 50 nm/min scanning rate, 2 s response time, 1 nm bandwidth as the average of 8 scans on a JASCO 810 spectrometer (Jasco, Inc.). For equilibrium measurements, the protein solutions were prepared at 0.2 mg/mL in 20 mM pH 4.6 phosphate buffer and measured in a 1 mm path length quartz cuvette (Starna, Inc) at room temperature. All sample spectra were corrected by subtraction of the corresponding spectrum of the buffer. Fractional secondary structure was estimated using *Selcon3* with the SP43 reference set as contained in the CDPro programs (accessible on-line via: http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [36].

2.4. Fluorescence

Fluorescence spectra were measured on a Fluoromax-3 spectrofluorometer (Horiba Jobin Yvon Inc.) with the samples held in a quartz cell (1 mm × 1 cm). The excitation wavelength was 295 nm and the emission spectra were collected from 300 nm to 500 nm. All the spectra were corrected by subtraction of the buffer spectrum.