SHEDDING LIGHT ON PROTEIN FOLDING DYNAMICS WITH HETEROGENEOUS UNFOLDED STATES

Spontaneous folding dynamics initiated by external electron transfer in equine heart cytochrome was monitored by time-resolved X-ray scattering. The systematic kinetic and structural analyses for the time-resolved data reveal that various protein conformations in the unfolded state take complex folding pathways, resulting in stretched exponential kinetics.

Protein undergoes a folding pathway to find a three-dimensional structure that expresses its biological function in a living cell. From vast theoretical studies, the funnel-like free-energy landscape for naturally avoiding Levinthal's paradox has been recognised as a key scheme for explaining the internal energies and conformational heterogeneities in protein folding **[1]**. Despite numerous experimental efforts, however, studies connecting protein folding and the conformational heterogeneity in the funnel-like energy landscape have been rare even for small model proteins. Thus, directly observing the evolution of such structural heterogeneity is highly desirable for an in-depth understanding of protein folding at a molecular level.

To achieve this goal, time-resolved X-ray solution scattering (TRXSS), also known as time-resolved X-ray liquidography (TRXL), was performed at beamline **ID09** to monitor the real-time evolution of unfolded equine heart cytochrome c (cyt-c) during folding. Cyt-c is a small metalloprotein with 104 amino acids and a heme group that is covalently bound to the protein backbone. Since the redox state of the heme acting as a cofactor is highly coupled with the folding free energy, it is possible to initiate spontaneous folding of cyt-c under certain denaturing conditions. Namely, the protein is fully unfolded in its oxidised state while it is fully folded in its reduced state **[2]**.

For the TRXSS study of cyt-c folding, the oxidised cyt-c with the unfolded conformation under a denaturing condition of 3.5 M GdnHCl was reduced by laser-induced electron transfer with nicotinamide adenine dinucleotide (NADH). The rapid photo-reduction subsequently initiated the folding process of cyt-c (**Figure 48**). This process was then tracked by X-ray probe pulses in the time domain ranging from early microseconds to late milliseconds (**Figure 48**). From the kinetic analysis of the time-resolved scattering data, it was possible to identify the stretched exponential kinetics in the transition of the early, unfolded intermediate to the folded state, which is sometimes referred to as strange kinetics **[3]**. This feature observed in TRXSS is in stark contrast to the normal exponential behaviour reported from earlier spectroscopic studies, which interpreted it as sequential transitions of homogenously populated intermediates. To obtain structural information from the X-ray scattering curve, an ensemble-based structural analysis was implemented with the aid of a molecular dynamics (MD) simulation. It showed

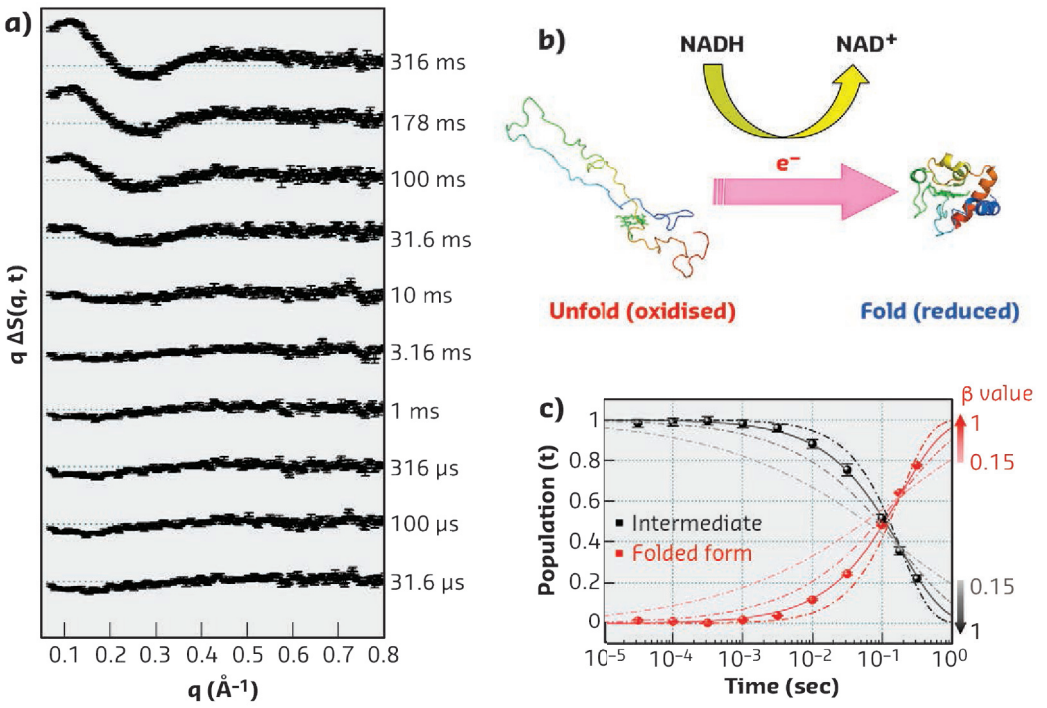

Fig. 48: a) TRXSS data for redox-coupled folding of cyt-c. **b)** Schematic of photo-induced electron transfer from NADH to oxidised cyt-c via solvated electrons. **c)** Stretched exponential kinetics in the folding of cyt-c determined from the analysis of TRXSS data. The β value is an indicator of how far the kinetics deviates from a normal exponential function with β = 1. The observed folding has a β = 0.70 and a relaxation time of 185 ms.

that the unfolded state is highly heterogeneous in terms of protein conformations. The combined results from the kinetic and structural analyses of X-ray scattering data reveal that the various protein conformations in the unfolded state take multiple relaxation pathways toward the native folded conformation, resulting in a stretched exponential behaviour (**Figure 49**). This observation directly reveals protein folding from heterogeneous unfolded states and exemplifies the strength of viewing microscopic protein conformations.

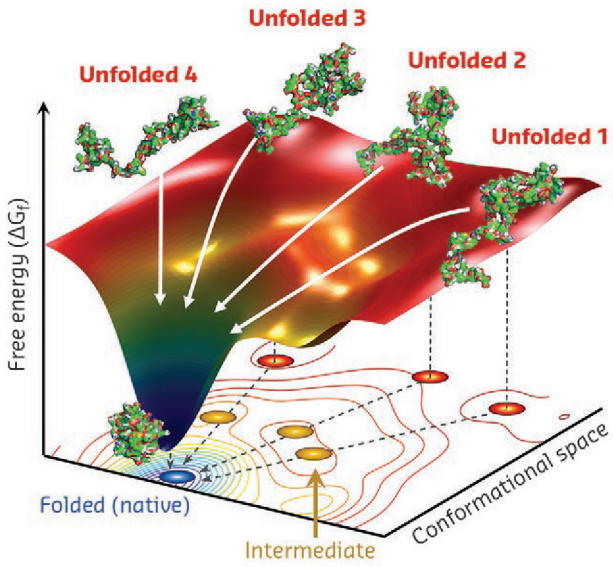

Fig. 49: Cyt-c folding dynamics in a funnel-like free-energy landscape. Upon the photo-reduction of oxidised cyt-c, the unfolded state with heterogeneous protein conformations (red dots in the projected plane) undergoes spontaneous folding along the multiple parallel pathways (white arrows) toward the native state with a homogeneously populated folded structure (blue dot in the projected plane).

This study is the first of its kind where the protein folding dynamics is investigated by viewing the conformational heterogeneity in relation to its kinetic behaviour. Thus, it should serve as a cornerstone for future studies of protein folding. One of the recent challenges in the fields of biochemistry and biophysics has been to obtain structural information from intrinsically disordered or unfolded proteins. In fact, understanding their biological roles should be founded on such conformational information. The TRXSS approach, combined with the systematic structural analysis at the conformational ensemble level, will make it possible to explore the structure-function relationship of such proteins and, potentially, their energetics at the molecular level.

PRINCIPAL PUBLICATION AND AUTHORS

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VITRIFICATION KINETICS VERSUS MICROSCOPIC DYNAMICS

Understanding the vitrification process plays a central role in commercial applications of glassy materials. XPCS reveals that the conventional wisdom based on a one-to-one correlation between the vitrification kinetics and the α-relaxation does not hold in all conditions; rather these two aspects of glassy dynamics are decoupled at deep undercooling.

In this work, a bulk metallic glass (BMG) based on an 18 k gold alloy was used to investigate the kinetics of vitrification of glass-forming systems. Contrary to polymeric and molecular systems, BMG-forming liquids do not show any reorientational or intramolecular motion that could influence their vitrification and atomic motions and, therefore, are ideal candidates for the study of these phenomena. The freezing process was studied in an unprecedentedly large range of cooling rates using a fast-scanning calorimeter (FSC) that allows extremely high cooling rates, avoiding crystallisation during the cooling from the melting temperature down to the glass transition.

Conventional wisdom held that the temperature dependence of the rate at which a liquid freezes and forms a glass should be equal to the rate at which the α-relaxation process decreases as the temperature is decreased. The α-relaxation is well described by the intermediate scattering function, which monitors the temporal evolution of the normalised density–density correlation