PROTEOMICS-LEVEL IDENTIFICATION OF KINETICALLY STABLE PROTEINS BY DIAGONAL 2D SDS-PAGE

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Introduction

Kinetic stability is a poorly understood property of a select group of naturally occurring proteins that are kinetically trapped in their native conformations (*1*). Kinetic stability can be best explained by illustrating the unfolding process as a simple equilibrium reaction between two protein conformations, the native folded state (N) and the unfolded state (U), separated by a transition state (TS) (Fig. 1). Since the height of the TS free energy determines the rate of folding and unfolding, kinetically stable proteins possess a TS with an unusually high free energy, resulting in extremely slow unfolding rates that virtually trap the protein in its native state (Fig 1A). Even when the overall change in Gibbs free energy (ΔG) may be favorable for unfolding under extreme solvent conditions, such as high concentrations of denaturant, the high activation energy of the TS significantly slows down the unfolding rate. It has been suggested that the presence of a high energy barrier separating the folded and unfolded states is an evolutionary feature to allow proteins to maintain activity in the extreme conditions they may encounter *in vivo* (*1*). This is consistent with the observation that thermodynamic stability alone does not fully protect proteins that are susceptible to irreversible denaturation and aggregation arising from partially denatured states that become transiently populated under physiological conditions (*2*). Therefore, the development of a high-energy barrier to unfolding may serve to protect susceptible proteins against such harmful conformational "side-effects". The physical basis for kinetic stability is poorly understood and no structural consensus has been found to explain this phenomenon. It appears that no single structural feature exists to explain kinetic stability, and perhaps this property may be achieved by different means, depending on the individual protein.

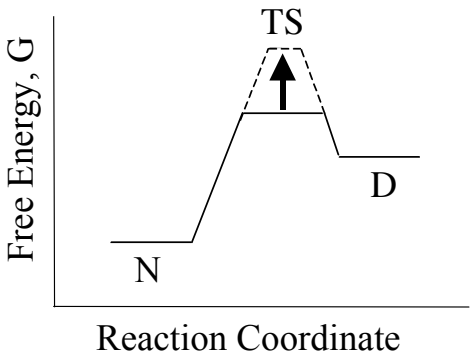

Fig. 1. Free energy diagram to illustrate the higher unfolding energy barrier for a kinetically stable protein under native conditions, as compared to that of a normal protein (represented by the dash line).

How to Analyze Protein Kinetic Stability?

The concept of kinetic stability as an alternative explanation for protein stability, independent from thermodynamic stability, was introduced in the early 90's (*3, 4*). Since then, kinetic stability has been shown to be an important physical property of some proteins (*5-7*). Since kinetically stable proteins are characterized by having a large energetic barrier that trap them in their native state (Fig. 1), determining the protein unfolding half-life under native conditions provides a quantitative description of kinetic stability. These experiments require pure protein and a fluorescence spectrometer/stopped-flow instrument to measure the rate of protein unfolding at different concentrations of denaturant (e.g. urea). The log of the unfolding rate is plotted against the denaturant concentration and the extrapolation of the linear plot to 0M denaturant yields the unfolding rate under native conditions. Thus, the small percentage of

proteins that are kinetically stable combined with the lack of a simple method to detect and quantify kinetic stability has limited the study and understanding of this important property of proteins.

SDS Resistance: A New and Simple Method to Screen For Kinetic Stability

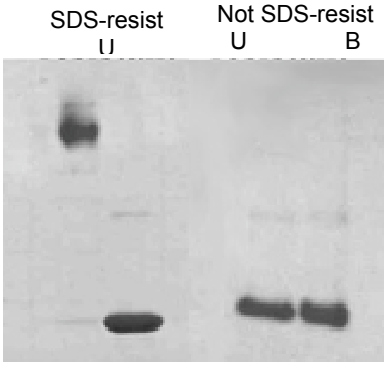

Fig. 2. 1D SDS-PAGE assay for identifying kinetically stable proteins. The protein is incubated in sample buffer (1% (w/v) SDS), divided in two, and one of them is boiled. The migration on the gel of the unboiled (U) and boiled (B) samples are compared. The "U" sample of kinetically stable proteins will migrate less than the "B" sample, while for SDS-labile proteins, both samples will have the same migration.

Manning and Colón discovered that kinetically stable proteins are resistant to denaturation by the detergent sodium dodecyl sulfate (SDS) (*8*) and this has led to the development of a simple boiled-unboil assay (Fig. 2) for detecting kinetic stability in proteins (*8*). Recently, we developed and optimized a diagonal 2-dimensional (D2D) SDS-PAGE version of the assay that preserves the key unboil-boil step to allow the screening of complex mixtures of proteins, such as cell lysates (*9*). Only microgram amounts of sample are needed, and the method is suitable for identifying kinetically stable proteins present in cell extracts without need for purification, thus making it a fast and inexpensive method for the detection of kinetic stability in proteins.

The concept behind our D2D SDS-PAGE method is illustrated in Fig. 3. Most proteins are easily denatured by SDS even without heating, and thus migrate the same distance in both dimensions of the gel analysis, resulting in a diagonal line of spots across the gel. However, SDS-resistant proteins travel a shorter distance in the 1st dimension due to minimal SDS binding. In the 2nd dimension, the SDS-resistant proteins become denatured by the heating step and exhibit the expected migration on SDS-PAGE, and consequently are located off the diagonal. We applied our D2D SDS-PAGE method to the cell lysate of *E. coli* and, as expected, the 2D gel showed a strong diagonal indicating that most proteins in *E. coli* are not kinetically stable (9). Proteomics analysis involving LC-MS/MS led to the identification of 50 non-redundant proteins that were SDS resistant (i.e. kinetically stable). Structural and functional analyses of a subset (44) of these proteins with known 3D structure revealed some potential structural and functional biases towards and against kinetic stability. This simple D2D SDS-PAGE assay should allow the widespread investigation of kinetic stability, including the proteomics-level identification of kinetically stable proteins in different systems, potentially leading to a better understanding of the biological and pathological significance of this intriguing property of proteins.

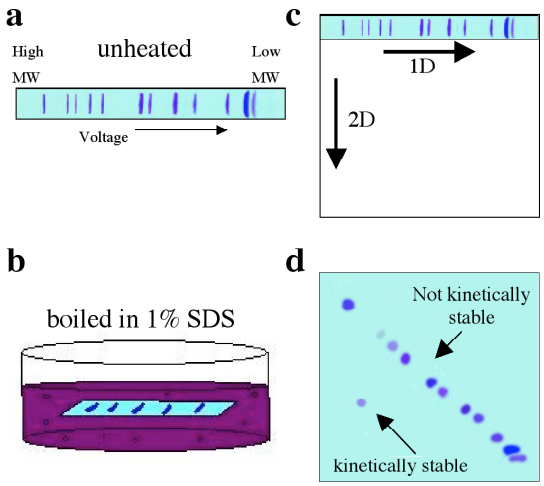

Fig 3. Illustration of the diagonal 2D SDS-PAGE procedure. **a)** The 1st dimension SDS-PAGE separation is performed. **b)** The lane is excised and incubated in boiling sample buffer containing 2% SDS. **c)** The proteins on the gel strip are transferred onto a larger gel and a 2nd dimension SDS-PAGE separation is performed. **d)** The non-kinetically stable proteins migrate equal distances in both SDS-PAGE dimensions to form a diagonal pattern, while kinetically stable proteins will migrate less in the 1st dimension, giving rise to spots that will fall below the gel diagonal. Figure is from reference 9.

References

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