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The pH dependence of staphylococcal nuclease stability is incompatible with a three-state denaturation model

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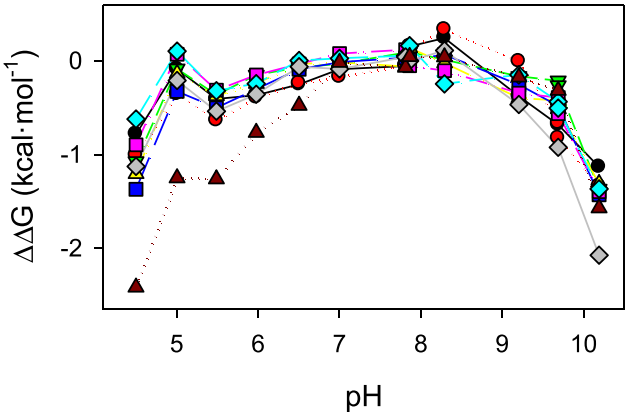
HIGHLIGHTS

- Six single mutations were made in a highly stable triple mutant of nuclease.
- Stabilities were measured in 13 buffers ranging over pH 4.50 to 10.19.
- m_{GuHCl} and ΔG_{H_2O} vary widely with pH, but differences between mutants do not.
- Therefore, most nuclease mutants do not denature by a three-state mechanism.
- Changes in m_{GuHCl} upon mutation do not arise from a three-state mechanism.

GRAPHICAL ABSTRACT



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ABSTRACT

Six single substitution mutations, V66F, V66G, V66N, V66Q, V66S, V66T, and V66Y, were made in the background of a highly stable triple mutant (P117G, H124L, and S128A) of staphylococcal nuclease. The thermodynamic stabilities of wild type staphylococcal nuclease, of the stable triple mutant and of its six variants were determined by guanidine hydrochloride denaturation in thirteen different buffers spanning the pH range 4.5 to 10.2. Within experimental error the values of ΔG_{H_2O} and m_{GuHCl} for the various proteins measured over this wide range of pH maintain a constant offset from one another, tracing a series of approximately parallel curves. This data offers an independent means of determining the error of stabilities and slopes determined by guanidine hydrochloride denaturations and shows that previous error estimates are accurate. More importantly, this behavior cannot be reconciled with a three-state denaturation model for staphylococcal nuclease. The large variations in m_{GuHCl} observed in these mutants must therefore arise from other causes.

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1. Introduction

We have published a number of studies [1–9] examining the effects of burying in the hydrophobic core residues which are normally ionized at neutral pH. These substitutions were done originally at position 66 of staphylococcal nuclease, a protein much used as a model system to study protein stability. The free energy differences

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between the native and denatured states of these proteins were examined as a function of pH to determine the apparent pK_a values of the ionizable groups in the denatured and native states. To extend the pH range over which protein stability could be measured, we made these mutations in a background of three other stabilizing mutations.

Because analysis of the pH dependence of stability to extract apparent pK_a values makes assumptions about the denaturation processes used to measure stability, it was of interest to examine the effects of pH on the stability of variants with substitutions other than ionizable groups at hydrophobic core positions. We focused on a highly stable variant of staphylococcal nuclease engineered with three stabilizing mutations. A set of control proteins were made with a number of other non-ionizable mutations at position 66, and we examined how their energetics varied with pH. We report here the results of our study and the surprising conclusion that mutants with widely varying stabilities and differing values of m_{GuHCl} appear to have very similar denatured states, a result in conflict with much of the literature regarding staphylococcal nuclease.

There is good evidence that some mutants of nuclease, most notably the V66W variant, have a well-populated equilibrium unfolding intermediate [10–22]. Some have argued most or all nuclease mutants as well as wild-type denature via an intermediate [23,24], meaning that a three-state model should be used to analyze the data rather than the two-state model commonly used. If wild-type and the many other mutants of nuclease characterized over the years do in fact denature via an intermediate state, a failure to take this into account in the analysis could lead to large errors in the apparent stabilities relative to the true free energy difference between the native state and the denatured states and could account for the variation in m_{GuHCl} between various substitution mutants of the protein.

Further complicating matters, Bolen's group has proposed that neither a simple two or three state model fully explains the data for wild-type nuclease and the majority of mutants that have been studied [22,25]. They argue for the variable two state model proposed by Shortle [26,27], in which the character of the denatured state changes with mutation, denaturant concentration, or temperature. However, they point out several disagreements between this model and experimental data [21,22,25].

2. Experimental procedures

2.1. Mutagenesis and protein expression

Since the stability of nuclease is too low to give reliable data at extremes of pH, the mutants were made in a GLA background. GLA is a hyperstable variant of nuclease that contains the mutations P117G, H124L, and S129A [28]. Originally, Shortle's group cloned nuclease [29] from the Foggi strain of *Staphylococcus aureus*, which differs from the nuclease in the V8 strain at position 124, which is L in V8 and H in the Foggi strain. Therefore either one of these residues at 124 might be regarded as wild type.

All mutations were introduced into the DNA sequence of GLA nuclease in a M13 vector using the method of Kunkel [30]. Protein expression and purification were carried out as previously described [31]. Final dialysis was against a 100 mM NaCl, 25 mM sodium phosphate buffer, pH 7.0. Purity was verified by SDS-PAGE. Typical protein yields were in the range of 5–15 mg of at least 98% pure protein.

2.2. Preparation of buffers

The mutants were first titrated with 6 M GuHCl that was buffered using pH 7.0 25 mM sodium phosphate, 100 mM NaCl. After determining that the mutant proteins in the GLA background had a stability of that of the corresponding mutant in the wild-type background plus 3.3 kcal·mol^{-1} (the difference between the stabilities of GLA and wild-type), they were then titrated with guanidine hydrochloride in other buffers over a wide range of pH.

The buffer used for pH values ranging from around 4.5 to 7.8 consisted of 25 mM bis–tris-propane (1,3-bis(tris[hydroxymethyl] methylamino)propane, Sigma) brought to the correct pH with acetic acid. Initially stock solutions of buffer were made at 100 mM concentration. These stock solutions were then diluted to 25 mM and the pH of the dilute solution was checked at the concentration intended for actual use. The buffer used for pH values ranging from around 7.9 to 9.7 consisted of 25 mM bis–tris-propane brought to the correct pH with phosphoric acid. The buffer used for pH values ranging from around 9.2 to 10.2 consisted of 25 mM ethanolamine from Sigma brought to the correct pH with hydrochloric acid. It should be noted that, in contrast to our regular procedure, no NaCl was added to the buffer. All buffers were filtered with Corning disposable sterile bottle top filters with a 0.22 μm cellulose acetate membrane to remove any suspended particles. The pH of the buffers was checked with a Beckman 39536 glass body combination calomel electrode and an Orion model 720A pH meter with a resolution of 0.001 pH units.

2.3. Preparation of buffered guanidine hydrochloride

6.00 M guanidine hydrochloride (GuHCl, Gibco Ultrapure grade) was prepared over the same range of pH at matching pH values for each 0.025 M buffered solution. GuHCl was added to a carefully weighed volume of the 100 mM stock buffer solution and an appropriate amount of water to bring the solution to approximately 25 mM buffer and 6 M GuHCl. The pH was first checked to see that it matched the original buffer. The density of the resulting buffered GuHCl solution was then checked and adjusted if necessary by adding either 25 mM buffer or GuHCl as appropriate to bring the concentration to ~6 M. Final adjustments were made by comparing the refractive index of the 25 mM buffer to that of the guanidine solution [32], using a Bausch & Laumb model 33-45-58 refractometer. In the case of buffered solutions at pH values 5.5 and less, the GuHCl concentration of the final solution was checked using density measurements alone due to difficulties with measuring the refractive index in those buffer systems. A comparison of density measurement with refractive index measurement at other pHs showed that the two different methods led to [GuHCl] within 0.02 M of each other.

2.4. Titration of proteins

The mutant proteins were titrated with buffered 6.00 M GuHCl as generally described previously [33]. The shifting of pH values during protein titration was measured as the 6.00 M GuHCl/0.025 M buffer solution was added to a quartz cuvette containing 3 mL of 0.025 M buffer plus 25 μg of wild type protein. Protein was added by weighing out a stock solution of protein of known concentration previously prepared in 25 mM sodium phosphate, 100 mM NaCl, pH 7.0. The volume of protein in phosphate/NaCl buffer added to each 3 ml of buffer was on the order of 15–30 μL. This simulated protein titration was carried out with the Beckman 39536 glass body combination calomel electrode in place and the stir bar spinning. The guanidine hydrochloride solution was added incrementally until the final concentration of GuHCl within the cuvette was in excess of 2 M. Fluorescence was also measured and compared to an identical setup minus the pH electrode in order to ensure that complete mixing was taking place inside the cuvette. The pH of the solution in the cuvette was checked with a Beckman 39536 glass body combination calomel electrode. The pH of the buffer plus GuHCl solution in the cuvette was also monitored for a mock titration in each buffer to in order to observe any pH shifting which might occur.

In preparation for titration an aliquot of the concentrated protein stock was placed in a corresponding buffer for which the pH has

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