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The unfolding pathways of the native and molten globule states of 5-aminolevulinate synthase

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ABSTRACT

In this communication, we report the equilibrium and kinetic properties of the unfolding pathways of the native (pH 7.5) and alkaline molten globule (pH 10.5) states of the pyridoxal 5′-phosphate (PLP)-dependent enzyme 5-aminolevulinate synthase (ALAS). The stability of the molten globule state is adversely affected by thermal- and guanidine hydrochloride (GuHCl)-induced denaturation, and the equilibrium unfolding pathways, irrespective of pH, cannot be described with simple two-state models. Rapid kinetic measurements, in the presence of denaturing GuHCl concentrations, reveal that at pH 10.5, the rate of ALAS denaturation is 3 times faster than at pH 7.5. From pH jump experiments, comparable rates for the denaturation of the tertiary structure and PLP-microenvironment were discerned, indicating that the catalytic active site geometry strongly depends on the stable tertiary structural organization. Lastly, we demonstrate that partially folded ALAS tends to self-associate into higher oligomeric species at moderate GuHCl concentrations.

1. Introduction

Molten globular proteins lack a well-defined tertiary structure, due to loosened packing of the side chains in the protein core, and form highly dynamic conformational ensembles [1]. Their secondary structure content, however, remains native-like and their protein fold is relatively compact [1]. The equilibrium molten globule state of murine erythroid-specific 5-aminolevulinate synthase (mALAS2), a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the initial step of heme biosynthesis [2], can be stabilized under acidic (pH 1 and pH 3 at 20 °C) and mildly alkaline (pH 10.5 at 20 °C and pH 9.5 at 37 °C) conditions [3]. Under these conditions, mALAS2 retains significant secondary structural content, but its tertiary structure rigidity is disrupted with hydrophobic surfaces becoming increasingly solvent-exposed. The mALAS2 fold remains compact in alkaline solution, while mALAS2 tends to self-associate into higher oligomeric species at acidic pH [3]. Interestingly, at pH 9.5 and 37 °C, mALAS2 is catalytically active, although the k_{cat} of the reaction is significantly lower than at physiological pH [3].

Previously we demonstrated that conversion of mALAS2 into the molten globule state modulates its structural organization and catalytic properties [3]; however, we did not address how pH-induced perturbations affect the stability and folding pathway of the enzyme. Here, we focus on the molten globule state of mALAS2 stabilized at pH 10.5 and 20 °C. Our data reveal that increased alkalinity of the mALAS2 milieu adversely affects its stability against thermal and guanidine hydrochloride-induced denaturation, and that the unfolding pathway is a complex multi-step process, with detectable aggregates formation at moderate denaturant concentrations.

2. Materials and methods

2.1. Protein purification

ALAS was purified as described [4]. The concentrated protein (7.3 mg/mL) was dialyzed against 50 mM phosphate buffer, pH 7.5 (36 mM K_2HPO_4/14 mM KH_2PO_4) containing 40 μM PLP at 4 °C in four

Abbreviations: ALAS, 5-aminolevulinate synthase; mALAS2, murine erythroid-specific 5-aminolevulinate synthase; PLP, pyridoxal 5′-phosphate; ANS, 8-anilino-1-naphtalenesulfonic acid; GuHCl, guanidine hydrochloride.

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successive exchange volume (0.5 L) steps. Aliquots were stored at −80 °C.

2.2. GuHCl- and thermal-induced denaturation studies

GuHCl-induced denaturation was monitored by following changes in fluorescence emission maxima upon excitation of the intrinsic protein fluorescence at 280 nm, using a 0.3 cm-path length cell. Unfolding reactions, performed in duplicates, were conducted at 20 °C in 50 mM phosphate, pH 7.5 (36 mM K_2HPO_4/14 mM KH_2PO_4) or 50 mM CAPS, pH 10.5 by equilibrating wild-type mALAS2 (1.2 μM) with various concentrations of GuHCl for 4 h. Equilibrium was established during this period. Stock solutions of 8 M GuHCL were prepared in either 50 mM phosphate or 50 mM CAPS, and their pH adjusted to 7.5 and 10.5, respectively. For experimental details on thermal denaturation, see Supplementary Material.

2.3. Circular dichroism (CD) studies

For experimental details, see Supplementary Material.

2.4. Stopped-flow spectroscopy

The kinetic measurements were conducted using a KinTek stopped-flow apparatus (model SF-2001) at 20 °C. For pH jump experiments, one syringe contained 60 μM mALAS2 in 10 mM K_2HPO_4, pH7.5, while the other syringe 250 mM CAPS, pH 10.5. The GuHCl perturbation experiments were conducted at pH 7.5 and pH 10.5. One syringe contained 60 μM mALAS2 in either 50 mM phosphate, pH 7.5 (36 mM K_2HPO_4/14 mM KH_2PO_4) or 250 mM CAPS pH 10.5, and the other syringe 8 M GuHCL prepared in either 50 mM phosphate pH 7.5 or 50 mM CAPS pH 10.5. A band pass filter (Andover corporation #340FS25-25) with a center wavelength at 340 nm was used to limit the fluorescence emission above this wavelength from reaching the detector upon excitation at 280 nm. For the pH jump experiments, we also monitored absorbance at 410 nm. Kinetic traces data were fit to one- or two-exponential equations [5], with each trace representing the average of at least 6 accumulated measurements.

2.5. Dynamic light scattering

Dynamic light scattering was measured at 25 °C using a Wyatt DynaPro plate reader. The protein concentration was 0.7 mg/mL. All experiments were done in either 20 mM phosphate pH 7.5 (16 mM K_2HPO_4/4 mM KH_2PO_4) or 20 mM CAPS pH 10.5 and with the following GuHCl concentrations: 0, 1.1, 2.2, and 4.9 M. Prior to data collection, all buffers and protein samples were filtered through a 0.22 μM filter, and the samples with the above stated denaturant concentrations were incubated for 3 h at 25 °C. Each measurement at a specific concentration of GuHCl was corrected to include the appropriate refractive index and viscosity. The refractive indexes were determined using an Atago refractometer, while GuHCl viscosities were from Ref. [6].

2.6. Reaction with ANS and acrylamide quenching of intrinsic protein fluorescence

For experimental details, see Supplementary Material.

3. Results and discussion

3.1. Equilibrium thermal and GuHCl denaturation profiles

Thermal stabilities of mALAS2 at pH 7.5 and pH 10.5 were characterized by monitoring the changes in ellipticity at 223 nm upon temperature increase (Fig. 1A). The alkalinity increase of the solution adversely affected the enzyme thermal stability, as evident from the ~16 °C reduction between the thermal transition midpoint (T_m) values at pH 10.5 (T_m = 33.15 ± 0.19 °C) and pH 7.5 (T_m = 49.67 ± 0.08 °C; from Ref. [7]). Furthermore, the pre-transition baseline of the unfolding curve at pH 10.5 is significantly diminished (Fig. 1A), implicating reduction in the cooperativity of the thermal transition. Since molten globular proteins are often described by non-cooperative melting transitions [1], this is yet another indicator that under alkaline conditions, mALAS2 populates the molten globule state. We note that at pH 7.5 and pH 10.5 the enzyme thermal denaturation was not fully reversible.

The GuHCl-induced denaturation of mALAS2 at pH 7.5 and pH 10.5 was monitored by following changes in intrinsic protein fluorescence (Fig. 1B). Denaturation curves indicate that, under alkaline conditions, the enzyme undergoes unfolding at lower GuHCl concentrations (Fig. 1B). Specifically, at 1 M GuHCl, the total fraction of unfolded mALAS2 molecules corresponds to 0.12 and 0.29 at pH 7.5 and pH 10.5, respectively. As the denaturant concentration increases to 2 M, the fraction unfolded increases to 0.39 and 0.73 at physiological and alkaline pH values, while at 4 M GuHCl, the enzyme is mostly unfolded irrespective of pH (0.98 fraction unfolded). Thus, both GuHCl- and temperature-induced denaturation reduce mALAS2 stability at pH 10.5. We note that unlike previous unfolding studies of mALAS2 achieved by GuHCl-induced denaturation of the wild-type and circularly permuted enzymes, which included osmolytes and detergents in the unfolding buffer [8], we eliminated additives in the phosphate and CAPS buffers at pH 7.5 and pH 10.5, because osmolytes (e.g., glycerol and methylamines) can stabilize native-like conformations over the molten globule[2] and complicate comparisons among the unfolding pathways.

To probe if the structural transformation of the enzyme into the GuHCl-denatured state follows a two-state (all-or-none) or multi-step (with the accumulation of stable intermediates) process, we constructed phase diagrams by plotting the fluorescence emission intensity at 365 nm as a function of the intensity at 320 nm. A linear relationship for $I(\lambda_1) = f(I(\lambda_2))$ has been postulated to indicate that protein unfolding proceeds as an all-or-none transition, without the accumulation of stable intermediates [9]. Here, $I(\lambda_1)$ and $I(\lambda_2)$ represent the fluorescence emission intensities collected at two wavelengths at different slopes of the spectrum (e.g., 320 and 365 nm). In contrast, a non-linear relationship would indicate that the protein denaturation was a multi-state process, with the unfolding of the structural organization occurring in sequential steps [9]. Phase diagrams of the GuHCl-induced denaturation of mALAS2, at pH 7.5 and 10.5, demonstrate that the relationship $I(\lambda_1) = f(I(\lambda_2))$ is non-linear (Fig. 1C and D), strongly suggesting that mALAS2 unfolding, under physiological and alkaline conditions, cannot be described by a simple all-or-none transition. We conclude that the unraveling of the structural integrity of mALAS2 in the presence of the chemical denaturant entails formation of stable intermediates or aggregates.

Because ALAS tends to aggregate in a concentration-dependent manner at low-to-moderate GuHCl concentrations (see section 3.6.), we did not refine the analysis of the denaturation equilibrium data (Fig. 1B). Analyses of equilibrium-unfolding curves for homodimeric proteins (e.g., mALAS2) with suitable three-state models require defining the oligomeric nature of the stable intermediate(s) [10]. This is routinely accomplished by analyzing the curves for protein concentration-dependency of the unfolding process, where

[2] Submitted manuscript by the authors.