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ATP-induced noncooperative thermal unfolding of hen lysozyme

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ABSTRACT

To understand the role of ATP underlying the enhanced amyloidosis of hen egg white lysozyme (HEWL), the synchrotron radiation circular dichroism, combined with tryptophan fluorescence, dynamic light-scattering, and differential scanning calorimetry, is used to examine the alterations of the conformation and thermal unfolding pathway of the HEWL in the presence of ATP, Mg^{2+}–ATP, ADP, AMP, etc. It is revealed that the binding of ATP to HEWL through strong electrostatic interaction changes the secondary structures of HEWL and makes the exposed residue W62 move into hydrophobic environments. This alteration of W62 decreases the β-domain stability of HEWL, induces a noncooperative unfolding of the secondary structures, and produces a partially unfolded intermediate. This intermediate containing relatively rich α-helix and less β-sheet structures has a great tendency to aggregate. The results imply that the ease of aggregating of HEWL is related to the extent of denaturation of the amyloidogenic region, rather than the electrostatic neutralizing effect or monomeric β-sheet enriched intermediate.

1. Introduction

The interaction between small molecules and proteins usually induces alterations in protein unfolding kinetics [1] (such as modifications in the melting temperature, enthalpy of unfolding, unfolding pathway, etc.) which has an important role in the amyloid aggregation of proteins [2]. Many studies have revealed that the process of amyloid aggregation could be affected by the presence of a significant quantity of charged polyelectrolytes [3,4]. In particular, ATP, a naturally occurring polyanion, dramatically increases the rate of amyloid aggregation of hen egg white lysozyme (HEWL) in elevated temperature and low pH [4–6], although it is considered to have no physiological role in the function of HEWL.

HEWL is known as a highly positively charged protein at low pH. Its interaction with the negatively charged ATP compensates the electrostatic repulsions between positively charged protein monomers [4]. However, a different effect of the electrostatic neutralization may exist as it is shown that ionic liquids could significantly inhibit the amyloid aggregation of HEWL [7]. HEWL comprises an α-domain containing several helices and a β-domain comprised largely of β-strands (Fig. S1). The amyloidogenic region of HEWL mainly encompasses part of the β-domain and Helix C of the α-domain [8]. Some studies suggest that monomeric β-sheet enriched intermediate is required for amyloid aggregation [9,10], and ATP might bind to these amyloidogenic precursors [11]. Nevertheless, no intermediate in amyloid aggregation of HEWL has been identified [7]. To date, the nature of the interactions and the degree of specificity involved in ATP-enhanced amyloidosis of HEWL are uncertain.

Recently, Morshedi et al. suggested that a putative interaction site in HEWL for indole derivatives [12] is the binding site of ATP [5], in which three tryptophan residues including W62, W63, and W108 are involved. Earlier studies reveal that the position alteration of W62 weakens the hydrophobic forces which hold the protein in its native structure [13–15]. W62 and W63 may first unfold in the HEWL unfolding [16]. In addition, W62G mutation first induces the unfolding of the β-domain of HEWL at high temperatures, and then the unfolding process spreads into the α-domain through Helix C [14]. Hence, whether the presence of ATP alters the W62 conformation or the unfolding pathway of HEWL must be key factors for understanding the nature and significance of the interactions between HEWL and ATP.

Here, synchrotron radiation circular dichroism (SRCD) and intrinsic tryptophan fluorescence are used to examine the conformational alterations of HEWL in the presence of ATP, Mg^{2+}–ATP complex (MgATP), ADP, AMP, and $MgCl_2$. SRCD and DSC are employed to characterize the thermal equilibrium unfolding of HEWL alone and in the presence of each additive. Moreover, dynamic light-scattering is used to probe the aggregation in the unfolding process. We for the first time observe an ATP-induced partially unfolded intermediate under the conditions of HEWL amyloid aggregation. The results imply that noncooperative unfolding of HEWL caused by ATP-induced perturbation of the residue W62, rather than the electrostatic neutralizing effect, is more critical for HEWL aggregation.

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2. Materials and methods

2.1. Materials

HEWL, AMP, ADP, and ATP (sodium salts) were purchased (Sigma). The other chemicals used were of analytical grade. All solutions were prepared with Mill-Q water. A HEWL stock solution of 20 mg/ml was prepared, and was extensively dialyzed against the solvent (aqueous solution of HCl at pH 2.0). AMP, ADP, and ATP solutions of 100 mM and $MgCl_2$ solution of 500 mM were prepared, and the pH was adjusted to 2.0 by addition of small amounts of 1 M HCl. $MgCl_2$ (10-fold) was mixed with isopyknic ATP solution to form MgATP. These stock solutions were stored at −80 °C. The samples were prepared by dilution from these stock solutions, and were centrifuged (16,000 rpm, 10 min). All sample cells filled with solution were sealed to keep the pH constant during the experiments. The accurate determination of protein concentration was as described [17]. For simplicity, in the following text, the HEWL in the presence of AMP, ADP, ATP, and MgATP are denoted by amp-HEWL, adp-HEWL, atp-HEWL, and mgatp-HEWL, respectively.

2.2. Synchrotron radiation circular dichroism (SRCD) experiments

The SRCD spectra were recorded from 25.0 to 70.0 °C on Beamline 4B8 in BSRF (Beijing, China) as described in [17]. All experiments were done in triplicate. The equilibrium time at each temperature was 5 min to ensure that three repeat scans exactly match each other. The same cell with a path length of 0.01 cm (Hellma) was used. The concentrations of HEWL and each additive used in samples were 0.14 and 1.40 mM, respectively. The protein was omitted from the control sample (baseline) of which the spectra at all temperatures were also recorded. The secondary structure contents were estimated by singular value decomposition (SVD) and the spectra set was deconvoluted using the convex constraint algorithm (CCA) [18]. DSSP was used to assign the secondary structure of HEWL in the crystal (PDB ID: 193L [19]) [20]. The SRCD performance, δ, was characterized by the root-mean-square deviation (RMSD) between the DSSP assignment and the SRCD estimates [21]. The quality of the fit of the calculated data to the experimental data was characterized by the normalized RMSD (NRMSD), whose values of <0.1 mean that they are in close agreement [22].

2.3. Intrinsic tryptophan fluorescence (ITF) experiments

The ITF spectra were monitored from 300 to 400 nm at 25 °C using a spectrofluorometer (Aminco-Bowman series 2) equipped with a temperature-controlled circulator bath. The excitation wavelength is 295 nm, and the excitation and emission bandwidths are both 4 nm. One hundred and fifty microliter portion of 7 μM HEWL was added to a cuvette with a path length of 5 mm in both the lateral and perpendicular directions. Each additive was then titrated into the HEWL solution in the cuvette. In the experiment of $MgCl_2$ back-titration, the sample of 7 μM HEWL in the presence of 7 mM ATP was added to the same cuvette, and $MgCl_2$ was then titrated. All baselines were measured in the same procedures.

2.4. Ultrasensitive differential scanning calorimetry (US-DSC)

DSC measurements were done with a VP-DSC microcalorimeter (MicroCal). All samples were the same with that used in the SRCD experiments. The reference cell and the sample cell were prewashed by the control and the sample, respectively. Each sample and the control were equilibrated at 10.0 °C for 15 min before heating. The scanning rate was 1.0 °C/min. After the first heating process, the cells were cooled. Then, the samples were again equilibrated at 10.0 °C for 15 min to eliminate the effect of thermal history before the second heating process.

2.5. Dynamic light-scattering (DLS)

DLS measurements were done from 25.0 to 60.0 °C with a DynaPro MSTC800 DLS instrument fitted with a 624.4 nm, 50 mW laser at a scattering angle of 90°. Fourteen micromolar of HEWL samples was prepared in the absence and presence of 140 μM each additive, respectively. All samples were filtered through a 100 nm membrane before the measurements. The hydrodynamic radius was analyzed using Dynamics V6.2 software.

3. Results and discussion

3.1. ATP-induced conformational alteration of the residue W62

In the ITF experiments, the emission maximum (EM) of 7 μM HEWL at pH 2.0 is around 338 nm. And the EM dramatically shifts to smaller wavelengths with the increasing of ATP concentration (Fig. 1A). However, ADP has much weaker effects, and no effect of AMP or Mg^{2+} is observed. Interestingly, the EM of 7 μM HEWL in the presence of 7 mM ATP gradually shifts back to 338 nm with the increasing of Mg^{2+} concentration (Fig. 1B); this should be because the negative charges of ATP phosphate groups which can chelate Mg^{2+} with very high affinity are neutralized. As a conclusion, the EM shifting to smaller wavelengths attributed to the movement of tryptophan residues to more compact, hydrophobic environments [23] is caused by the electrostatic effect of ATP phosphate groups and is strongly dependent on the density of negative charges.

The secondary structure contents of HEWL determined from the SRCD spectra at 25 °C are in good agreement with DSSP assignment, as indicated by the factor δ (Table 1). There is no apparent alteration in the SRCD signal of HEWL in the presence of MgATP, ADP, or AMP. However, the addition of ATP induces that the

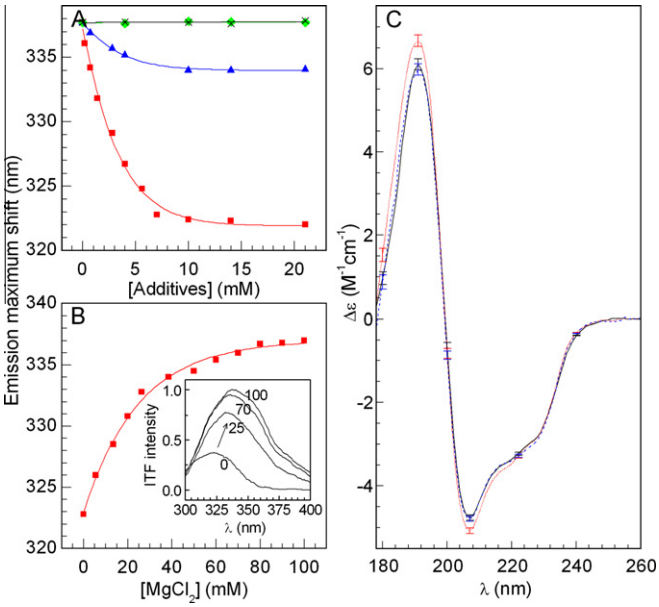

Fig. 1. The ITF and SRCD assays of HEWL in the absence and presence of different additives at 25 °C, pH 2.0. (A) The emission maximum shifts of the tryptophan fluorescence of HEWL produced by varying concentrations of ATP (*squares*), ADP (*triangles*), AMP (*diamonds*), and $MgCl_2$ (*crosses*). (B) $MgCl_2$ back-titration of atp-HEWL. The inset shows the fluorescence spectra marked with the concentrations of $MgCl_2$. (C) SRCD spectra of HEWL (*solid line*), mgatp-HEWL (*dash line*), and atp-HEWL (*dot line*). The error bars represent one standard deviation of the measurements from three sequential scans of both spectra and baselines.