THE JOURNAL OF PHYSICAL CHEMISTRY Letters

Letter

pubs.acs.org/JPCL

Probing the Acid-Induced Packing Structure Changes of the Molten Globule Domains of a Protein near Equilibrium Unfolding

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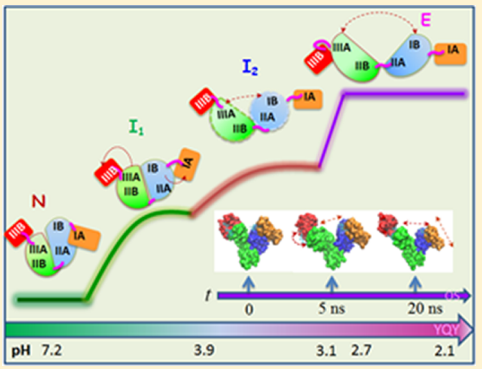
Supporting Information

ABSTRACT: Using simultaneously scanning small-angle X-ray scattering (SAXS) and UV−vis absorption with integrated online size exclusion chromatography, supplemental with molecular dynamics simulations, we unveil the long-postulated global structure evolution of a model multidomain protein bovine serum albumin (BSA) during acid-induced unfolding. Our results differentiate three global packing structures of the three molten globule domains of BSA, forming three intermediates $\mathbf{I_1}$, $\mathbf{I_2}$, and **E** along the unfolding pathway. The $\mathbf{I_1}$−$\mathbf{I_2}$ transition, overlooked in all previous studies, involves mainly coordinated reorientations across interconnected molten globule subdomains, and the transition activates a critical pivot domain opening of the protein for entering into the **E** form, with an unexpectedly large unfolding free energy change of −9.5 kcal mol^{-1}, extracted based on the observed packing structural changes. The revealed local packing flexibility and rigidity of the molten globule domains in the **E** form elucidate how collective motions of the molten globule domains profoundly influence the folding−unfolding pathway of a multidomain protein.



The residual tertiary structures of protein intermediates carry profound implications in understanding the protein folding−unfolding pathway or functioning.[1−8] Hydrophobic effects, resulting in a collapsed core of hydrophobic amino acids, are considered the major determinant of protein structural stability in aqueous solution.[3,8] Enhanced conformational entropy under environmental stimulations, however, competes with the van der Waals interactions for protein structural transitions toward functioning or folding−unfolding.[6−8] Recent progress has established a concept of molten globule domains for intrinsic disordered or partly unfolded proteins,[1−6] characterized by substantial secondary structure and a fluctuating tertiary structure with perturbed side-chain packing and hydrophobic cores (with or without core−water solvation).[3,8] The packing structure and melting sequence of the molten globule domains of a multidomain protein further influence the folding−unfolding pathway. Advances in molecular dynamics simulations have foreseen the dependence of folding−unfolding pathways of a multidomain protein on its topology of domain connectivity.[7,8] Nevertheless, experimental limitations have so far largely prohibited probing the global packing structures of the molten globule domains of multidomain proteins.

Probing the morphological evolution of protein intermediates has been challenging because of the generally less rigid structures that can blur or blind spectroscopic tools to different extents. These noncrystalline protein structures in solution exclude the use of powerful protein X-ray crystallography, whereas large and disordered structures often limit NMR sensitivity. Moreover, coexistence of protein intermediates further complicates the structural measurements and data interpretation. Nevertheless, optical spectroscopies including circular dichroism (CD), light absorption, fluorescence, and resonance Raman scattering are used to capture complementary local structural information on protein intermediates during folding−unfolding.[9,10] On the other hand, global structural conformations of native proteins in solution are increasingly better determined using small-angle X-ray or neutron scattering (SAXS and SANS).[11−16] Recent developments on combining SAXS with an online size exclusion high performance liquid chromatography (HPLC) column allow scanning along the elution path for SAXS data collection with online purified or separated protein species.[17] Rapid advance of model simulation with SAXS data further allows incorporation of local structural information (as fitting constraints) obtained from different structural probes in an integrated reconstruction of protein structure.[18] These developments have incubated a new approach that can break the conventional constraints on probing the packing structures and transitions of the molten globule domains in protein folding−unfolding pathways.

In this study, we perform scanning SAXS/UV−vis absorption measurements along the elution path of HPLC to probe the

Received: November 21, 2016
Accepted: January 3, 2017

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DOI: 10.1021/acs.jpclett.6b02722
J. Phys. Chem. Lett. 2017, 8, 470−477

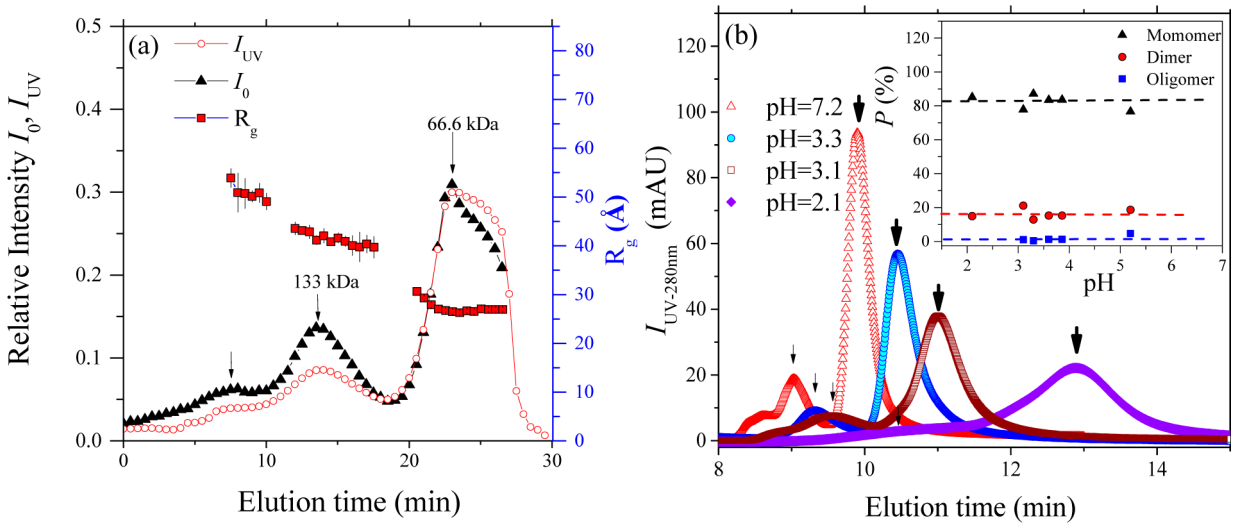

Figure 1. R_g and I_0 profiles extracted from the SAXS data measured along the chromatogram I_{UV} (280 nm wavelength) of a BSA solution at pH 7.2. The arrows mark the separated monomer, dimer, and oligomer peaks of BSA (molar mass selectively indicated). A slower HPLC elution rate was used for improved SAXS data collection time. (b) Representative chromatograms for BSA in the solutions at pH 7.2−2.1. The monomer and dimer elution peaks are marked with thick and thin arrows, respectively. The inset shows the molar fractions P extracted from the corresponding integrated peak areas of the species indicated.

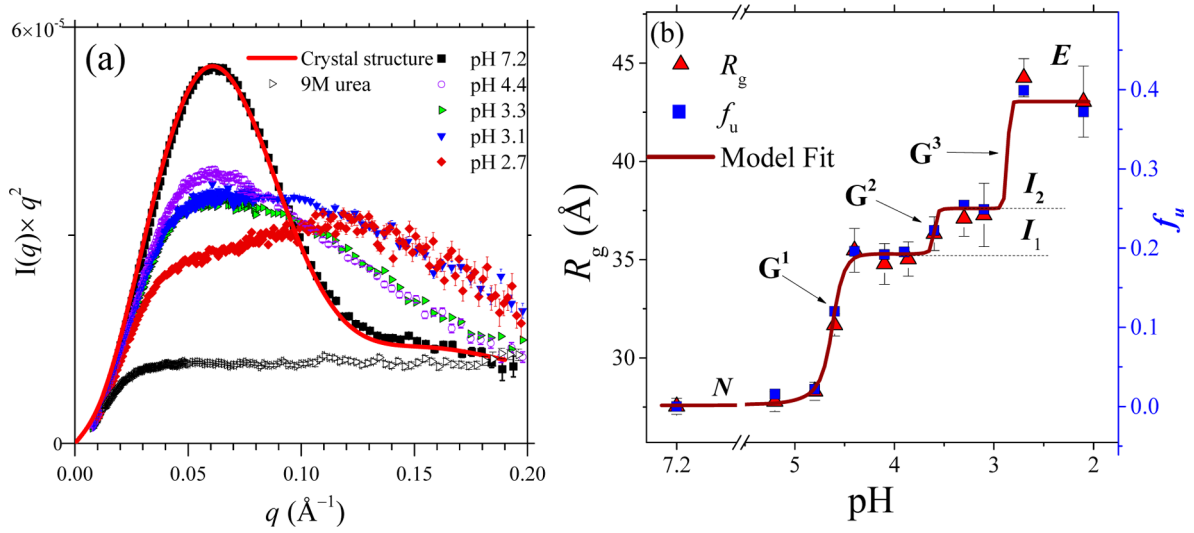

Figure 2. (a) pH-dependent SAXS data of BSA in the Kratky−Porod presentation. The SAXS data of the native form at pH 7.2 can be described well (solid profile) using the BSA crystal structure. SAXS data for an unfolded BSA with 9.0 M urea are shown for comparison. (b) Corresponding R_g values of BSA in the acid-induced unfolding. The unfolding fraction profile f_u (Y-axis to the right-hand-side) deduced from the protein unfolding dimension D_v is fitted (solid curve) using the modified Ising model with the three-group unfolding $\mathbf{G}^1$, $\mathbf{G}^2$, and $\mathbf{G}^3$ indicated for the transitions from the native state (**N**) to the intermediates $\mathbf{I}_1$, $\mathbf{I}_2$, and **E**, as marked.

intermediates of a model protein bovine serum albumin (BSA) partially unfolded in acid solutions between pH 2.1 and 7.2. The heart-shaped serum albumin, a transport protein comprised of three major domains, is one of the most abundant circulatory proteins in mammal blood.[19] Due to the distinguished multidomain structure, serum albumin can conform for noncovalent associations with fatty acids, hemin, bilirubin, and drugs, as a carrier.[19,20] Such nature renders BSA one of the most extensively utilized proteins in laboratory practice, serving as a human serum albumin substitute on many occasions. Despite extensive applications, the structural complexity has delayed BSA crystal structure determination until lately.[21] On the basis of local structural features, earlier studies[22−28] established two intermediates of serum albumin in acid-induced unfolding, termed as the fast (**F**) and expanded (**E**) forms.[9,23−28] The global packing structure of the three molten globule domains that constitute the intermediates, however, remains postulated to date. Here, we show that the scanning HPLC-SAXS approach can effectively exclude scattering contamination from coexisting oligomer species and collect SAXS of individual protein intermediates along the HPLC elution path. Integrating local structural information in the SAXS-based structure modeling[29,30] (rigid components mixed with flexible loops of a protein), we further elucidate subtle coordinated reorientations across interconnected molten globule subdomains, leading to a cleft in the domain−domain interfaces, prior to a critical pivot opening of the domains into an expanded intermediate of BSA. Such stepwise structural

DOI: 10.1021/acs.jpclett.6b02722
J. Phys. Chem. Lett. 2017, 8, 470−477

transitions cannot be explained by the conventional two-state model with an unfolding fraction determined from the populations of coexisting folded and unfolded proteins. A recently modified Ising model[14] is, therefore, adopted to interpret the observed multistep structural changes in acid-induced BSA unfolding, in terms of a topological collection of folding−unfolding units that constitute a protein. The modified Ising model provides a unique view to interpret the multistep unfolding process of BSA observed and allow extraction of the corresponding unfolding free energy changes of the grouped folding−unfolding units.

Figure 1a illustrates the zero-angle intensity I_0 and R_g values extracted using the Guinier approximation[11,12] from the SAXS data measured along the HPLC elution path of the native BSA sample solution at pH 7.2 (Figure S1 in the Supporting Information (SI)). The successively differentiated peaks indicate coexisting species in the sample solution. The last two peaks, corresponding to calibrated molecular masses of ∼132 and 66 k Da, are assigned to BSA dimer and monomer, respectively. The corresponding R_g values, 40.0 ± 0.5 and 28.1 ± 0.5 Å, are consistent with those calculated from the crystal structures of BSA dimer and monomer (R_g = 40.0 ± 0.2 and 27.5 ± 0.1 Å, as shown in Figure S2).[21] Shown in Figure 1b are the representative chromatograms measured for the BSA solutions at pH 7.2−2.1, each exhibiting three similar elution peaks as those in Figure 1a. Interestingly, the integrated peak areas of the three species remain of the same (molar) ratio of 82:16:2 for the monomer/dimer/oligomer in the solutions regardless of the pH values (inset of Figure 1b), implying no transformation among the three species in the pH range. The BSA monomer elution peak, however, systematically delayed and broadened with the decrease of the pH values. Such behavior is attributed to gradually enhanced nonspecific interactions between the increasingly positively charged BSA and the silica-based chromatography matrix (of negative surface charge) in the acidic environments below the protein isoelectric point (ca. pH 5). A previous report suggested an effective charge of +21 e (or a net charge of +65 e, which is consistent with our molecular dynamics simulation result) for BSA at pH 2.4; in contrast, the native protein has an effective charge of −8 e (or a net charge of −11 e at pH 6.8).[31] Most importantly, the monomer peaks for the BSA solutions at pH 3.3, 3.1, and 2.1 are separable and overlap little with that of the native state at pH 7.2 in the elution time, as shown in Figure 1b, suggesting that there is little coexistence of the BSA intermediates with the native form in the solutions.

Figure 2a illustrates the scanning HPLC-SAXS data corresponding to the chromatographs shown in Figure 1b, in the Kratky−Porod presentation.[32,33] The bell-shaped profile centered at $q_c \approx 0.06$ Å^{-1} for the BSA solution at pH 7.2 reveals a globular structure, with an estimated R_g of ∼28 Å using $q_cR_g \approx 3^{1/2}$.[32] When the solution pH value is lowered to 2.7−2.1, a relatively smeared and extended hump covering a wide q range between 0.05 and 0.2 Å^{-1} implies an extended structure.[32−35] For BSA partially unfolded in solution pH 4.8−3.1, SAXS profiles transit gradually from the bell-shape to the extended shape. For comparison, SAXS data for an unfolded BSA with 9 M urea (Figure 2a)[34] exhibit a characteristic plateau region of random coil behavior in the higher-q regime (>0.05 Å^{-1}).[32,33] Exhibited in Figure 2b are all of the pH-dependent R_g values of the acid-unfolded BSA extracted from the SAXS profiles simulated with the best-fitted models (as representatively shown in Figure 3), which are consistent with those obtained

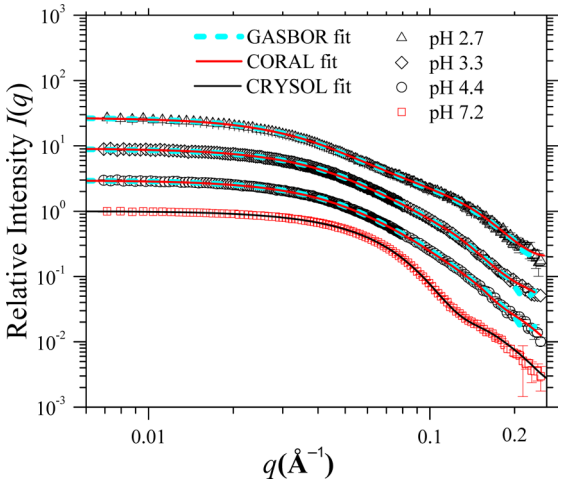

Figure 3. Representative SAXS data of BSA partially unfolded at the pH values indicated. The data are respectively fitted using the GASBOR (dashed curves) and CORAL (solid curves) models illustrated in Figure 4. The native BSA data (pH 7.2) are fitted (solid curve) using CRYSOL with PDB (4F5S).

using the Guinier approximation (Figure S3 and Table S1).[33] Concentration-independent SAXS profiles suggest little effect of interprotein interactions on these R_g values (Figure S4). These pH-dependent R_g values exhibit three-step transitions, beyond the one-step transition of the conventional two-state model, characterizing transitions from the native (N) to the $\mathbf{I}_1$, to the $\mathbf{I}_2$ intermediate, and then to the commonly recognized expanded **E** form. We note that our UV−vis absorption data simultaneously observed with the HPLC-SAXS at the same sample position reveal a smeared transition over $\mathbf{I}_1$ and $\mathbf{I}_2$ (Figure S5); previous CD and fluorescence results could not resolved these two intermediates $\mathbf{I}_1$ and $\mathbf{I}_2$ and smeared them as the **F** form.[36,37] The uniquely SAXS-revealed $\mathbf{I}_1$−$\mathbf{I}_2$ and $\mathbf{I}_2$−**E** transitions involve largely reorganization of the packing structure of the molten globule domains, which might be insensitive to local structural probes.

The multistep unfolding behavior of BSA shown in Figure 2b obviously cannot be interpreted using the convention two-state model, which assumes that the folded and unfolded proteins are populated according to a Boltzmann distribution determined by an unfolding free energy change and temperature of the system.[16] Moreover, the chromatographs in Figure 1b suggest little coexistence of **N** with the $\mathbf{I}_1$, $\mathbf{I}_2$, or **E** states in the acid-induced unfolding. Therefore, we adopt a modified Ising model recently developed for protein folding−unfolding that can describe the multistep unfolding behavior of BSA observed.[38−41] Briefly, the modified Ising model regards a protein as a topological collection (ensemble) of interacting units; each unit may take either the folded or unfolded state.[38−41] Both thermodynamic and near-equilibrium kinetic characteristics of protein folding−unfolding are assumed to be determined by the elementary process in folding−unfolding of the structural units inside the protein, expressed as the protein unfolded fraction f_u. When all units of a protein are in the folded (or unfolded) state, the protein behaves as fully folded with f_u = 0 (or unfolded with f_u = 1.0). The value of f_u can be between zero and unity for partially folded proteins, and all of the protein in the same environment can be in the same f_u of a common conformation, as revealed by our HPLC elution result. Furthermore, evolution of the protein morphology in a folding−unfolding process is

DOI: 10.1021/acs.jpclett.6b02722
J. Phys. Chem. Lett. 2017, 8, 470−477

Table 1. Thermodynamic Parameters Fitted with the Modified Ising Model for BSA in pH-Induced Unfolding[a]

group	γ	$2\Delta\varepsilon_m^I$ (kcal mol^{-1} M^{-1})	J^I (kcal mol^{-1})	ΔG_m^I (kcal mol^{-1})	ΔG_o^I (288 K) (kcal mol^{-1})	ΔG_J^I (kcal mol^{-1})
$\mathbf{G^1}$	0.19	$-(9.68 \pm 0.40) \times 10^4$	0.13 ± 0.01	-3.9 ± 0.2 (pH 4.4)	2.3 ± 0.1	-2.1 ± 0.2
$\mathbf{G^2}$	0.06	$-(1.96 \pm 0.44) \times 10^4$	0.28 ± 0.02	-9.8 ± 0.3 (pH 3.3)	4.9 ± 0.1	-6.0 ± 0.5
$\mathbf{G^3}$	0.14	$-(1.60 \pm 0.16) \times 10^4$	-0.13 ± 0.01	-32.0 ± 0.5 (pH 2.7)	21.9 ± 0.2	-9.5 ± 0.5

[a]The three folding−unfolding groups $\mathbf{G^1}$, $\mathbf{G^2}$, and $\mathbf{G^3}$ together contribute 39% (f_u) of the protein unfolding in D_v. The values for the free energy changes ΔG_m^I are deduced at 288 K. Note that the positive and negative J values of the three groups contribute respectively to the conformation instability and stability of the intermediates.

governed by the free energy changes of the folding−unfolding units of the protein in the modified Ising model and can have stepwise changes when the units are of distinct unfolding free energy changes. Thereby, the unfolding behavior of a protein containing multigroups (I) can be described by accumulated group unfolding $f_u = \sum_I \gamma_I f_u^I$, with the summation of the weighting coefficients γ_I (relative populations of the folding−unfolding unit) normalized to unity.

Under a mean-field approximation, the unfolded fraction of the Ith group of the modified Ising model is given by[38−41]

$$f_u^I = \left\{1 + \exp\left[\frac{\Delta G^I + 4J^I(1 - 2f_u^I)}{k_B T}\right]\right\}^{-1} \quad (1)$$

assuming that the canonical ensemble lies not far from thermodynamic equilibrium. In the above expression, only nearest-neighbor interactions (J) between folding−unfolding units in the same group are considered, and the unfolding free energy change of the Ith group is expressed as

$$\Delta G_J^I = \Delta G^I(C_m, T) + 4J^I(1 - 2f_u^I) \quad (2)$$

with $\Delta G_J^I = \Delta G_m^I + \Delta G_o^I(T)$ determined by the denaturant free energy change $\Delta G_m^I = 2\Delta\varepsilon_m^I C_m$ with the coefficient $2\Delta\varepsilon_m^I$ and the denaturant concentration C_m (in our case, the proton concentration), together with the thermal unfolding free energy change $\Delta G_o^I(T)$.[38−41]

We further propose an r^3-weighted protein dimension D_v as a measure of protein unfolding dimension, which is defined by the distance distribution function $p(r)$[11,42,43] Fourier transformed from the SAXS data as

$$D_v^3 = \frac{\int_0^{D_{max}} r^3 p(r)\, dr}{\int_0^{D_{max}} p(r)\, dr} \quad (3)$$

with $p(r)$ approaching zero smoothly at the maximum dimension $r = D_{max}$. We define the unfolding fraction profile as[40,41]

$$f_u = (D_v - D_{v,N})/(D_{v,N} - D_{v,U}) \quad (4)$$

with the minimum $D_{v,N}$ of the native state (at pH 7.2) and the maximum $D_{v,U}$ of the unfolded BSA (with the 9 M urea case). As shown in Figure 2b, the hence deduced unfolding fraction profile $f_u(D_v)$ of BSA can be satisfactorily described by a three-group unfolding of $\mathbf{G^1}$, $\mathbf{G^2}$, and $\mathbf{G^3}$, with the best fitted thermodynamics parameters shown in Table 1. These parameters suggest that a relatively larger group with γ^1 = 0.19 of $\mathbf{G^1}$ unfolds completely for the intermediate $\mathbf{I_1}$ when the pH value is ∼4.4, which is followed by the unfolding of a relatively smaller group $\mathbf{G^2}$ (γ^2 = 0.06) when the pH value reaches pH 3.3 for the intermediate $\mathbf{I_2}$. At pH 2.7, the third group $\mathbf{G^3}$ (γ^3 = 0.14) unfolds to the **E** form. With the three groups unfolded, the **E** form has an accumulated f_u = 0.39 (Figure 2b) and carries a substantial residual tertiary structure, as illustrated below. The corresponding group unfolding free energy changes of $\mathbf{G^1}$, $\mathbf{G^2}$, and $\mathbf{G^3}$ reinforce successively from ΔG_J^I = −2.1 to −6.0 to −9.5 kcal mol^{-1} (Table 1), in the stepwise multigroup unfolding. We note that the previously reported unfolding free energy change of ∼6.0 kcal mol^{-1} for BSA in a couple of occasions,[3,44] on the basis of local structural changes, likely corresponds to the unfolding free energy change ΔG_J^2 of the $\mathbf{G^2}$ group here. The significantly larger ΔG_J^3 = −9.5 kcal mol^{-1} of the $\mathbf{G^3}$ group unfolding (for the $\mathbf{I_2}$−**E** transition) revealed uniquely in our study can be attributed to large global packing structure changes associated with the pivot domain interactions of BSA, as detailed from the structural analysis below. We note that the proposed free energy changes for the respective transitions are based on the assumption of reversible transitions between the acid-induced intermediates and the native state, which is well supported by an observed refolding of BSA from the **E** form to the native state (Figure S6).

To reveal the corresponding packing structure changes of the molten globule domains in the three-step unfolding, we performed model simulations with the SAXS data. Our SAXS data analysis strategy is to obtain global protein envelopes of the intermediates using *ab initio* modeling of GASBOR for reference.[42] Guided by the best-fitted envelope morphology of the acid-induced intermediates of BSA, we further modeled the same set of SAXS data with the CORAL algorithm[43] that allows construction of structures from mixed molten globule domains (as rigid bodies) and flexible chains for an optimized conformation. In the CORAL structure modeling, we used an initial BSA structure model deduced from the original crystallographic BSA structure (PDB entry 4F5S)[21] and assigned the subdomains **A** and subdomains **B** (both are rich in antiparallel α-helices and with disulfide bonds between helices) in each of the three major domains **I**, **II**, and **III** of BSA as rigid domains in CORAL modeling. The subdomains are interconnected by long loops (12−13 dummy residues) to adjust the relative spacing and orientations.[21] After rigorous fitting attempts, we found that the fitting could be much improved with the CORAL rigid domains regrouped into Domain **IA** (residues 6−104), Domain **IB**−**IIA** (residues 118−291), Domain **IIB**−**IIIA** (residues 304−489), and Domain **IIIB** (residues 502−583), together with one free coil (5 residues in the N-terminal) and three flexible interdomain linkers for the four CORAL rigid domains. We further took into account the structural constraints that all of the local structures with the 17 disulfide bonds should be maintained in the acidic unfolding to restrict the freedom of nearby polypeptide chain movements.

Figure 3 shows the fitted SAXS data for **N**, $\mathbf{I_1}$, $\mathbf{I_2}$, and **E** states of BSA using the GASBOR and CORAL structural models

DOI: 10.1021/acs.jpclett.6b02722
J. Phys. Chem. Lett. 2017, 8, 470−477

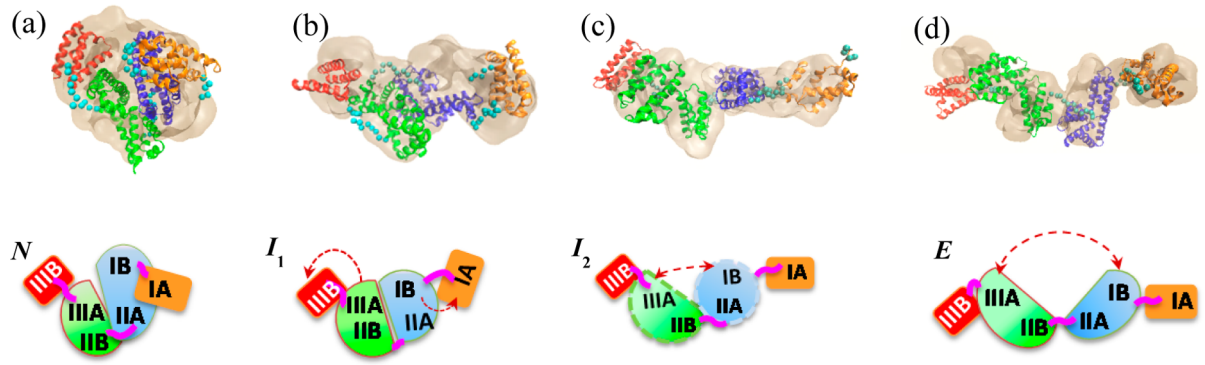

Figure 4. (a–d) CORAL models for the acid-induced unfolding of BSA at the states indicated. The small spheres represent the dummy residues of the flexible interdomain linkers between the four CORAL rigid domains (color-coded). Corresponding GABOR envelopes are shown in surface presentation for comparison. Corresponding cartoons illustrate the changes (dotted arrows) of the four assigned rigid domains interconnected by flexible chains (thick segments). The conventional assignments of the BSA major domains are marked with **I**, **II**, and **III**, each with **A** and **B** subdomains.

Table 2. SAXS Parameters for the BSA Solutions at the Selected pH Values[a]

state (pH value)	**N** (7.2)	$\mathbf{I}_1$ (4.4)		$\mathbf{I}_2$ (3.3)		**E** (2.7)	
$I(0)/c$ (cm^{-1} mg^{-1} mL)	2.42 ± 0.13	2.67 ± 0.13		2.72 ± 0.13		1.50 ± 0.13	
R_g (Å) of GA	27.5 ± 0.4	35.5 ± 1.1		37.1 ± 0.9		44.3 ± 1.0	
R_g (Å) of $p(r)$	27.6 ± 0.1	35.5 ± 0.1		38.1 ± 0.2		44.7 ± 0.3	
D_{max} (Å)	86.1	124.2		129.9		154.9	
fitting algorithm	CRYSOL	GASBOR	CORAL	GASBOR	CORAL	CORAL	EOM
fitting χ^2	1.21	1.28	1.01	1.00	1.00	1.60	1.53

[a]The zero-angle SAXS intensity $I(0)$ is scaled to the absolute intensity for 1.0 mg mL^{-1}, in units of cm^{-1} (cross section per unit volume).[50] Values for the maximum pair distance D_{max} are determined from the corresponding pair distance distribution functions $p(r)$ Fourier transformed from the SAXS data.[11,42,43] R_g values are extracted from the Guinier approximation (GA) and $p(r)$ functions.

shown in Figure 4. The best-fitted CORAL models overlap decently with the corresponding GASBOR envelopes (Figure 4 for the side views and Figure S7 for the top views). All extracted SAXS parameters are summarized in Table 2. These CORAL structural models reveal delicate transitions of the molten globule domains of BSA in the acid-induced unfolding. In the first unfolding step to the intermediate $\mathbf{I}_1$ from the native state, swing opening of the molten globule domains **IIIB** and rotation of **IA** (Figure 4b) complete the unfolding of the $\mathbf{G}^1$ group defined by the modified Ising model. Entering into $\mathbf{I}_2$ from the $\mathbf{I}_1$ state, however, involves a collective reorientation of the interconnected (**IIIA**–**IIB**) and (**IIA**–**IB**) domains (Figure 4c), forming a cleft in the pivot domain interfaces; this corresponds to the relative small fraction $\gamma \approx 6\%$ of the $\mathbf{G}^2$ group of the modified Ising model. Further transition to the **E** form at pH 2.7 or lower requires a follow-up pivot opening of the two interconnected domains (**IIIA**–**IIB**) and (**IIA**–**IB**), with the intersection of the **IIB** and **IIA** subdomains as the pivot (Figure 4d). The structural models in Figure 4 not only reveal the long-postulated packing structure evolution of the molten globule domains of the BSA intermediates but also characterize the structural transition sequence along the unfolding path. We also carefully checked local structural changes reported in many previous studies[9,22−28] and found that the available pieces of evidence are largely consistent with the three-step unfolding sequence proposed here (as detailed in the SI).

Previous NMR results suggested possible fluctuations of the two interconnected (**IIIA**–**IIB**) and (**IIA**–**IB**) domains in a similar acid-induced unfolding process.[45] In addition, hydrophobic solvation of the molten globule domains **I** and **II** for a softened structure of the **E** form was also reported lately.[3] These pieces of evidence suggest certain structure flexibility of the expanded **E** form. To further account for such structure flexibility in solution, we used the ensemble optimization method (EOM)[46] in search of an optimal structure ensemble that could best fit the same set of SAXS data (pH = 2.7). The EOM-generated structure pool for BSA contains 10 000 conformations, from the most compact ($R_{g\text{-}min}$ = 25 Å) to the most extended ($R_{g\text{-}max}$ = 55 Å) form. Moreover, the same constraints of the assignments of rigid domains and flexible chains used in the CORAL approach were also applied in the EOM model fitting with the same set of SAXS data. The best SAXS data fitting (χ^2 = 1.53; see Figure S8) was achieved with an ensemble comprising six equal-probable structural models representatively shown in Figure 5 (completely shown in Figure S9). We note that the resolution of the size exclusion column (ca. 6 Å in R_g) used in our SAXS data collection helped to reject certain ensembles containing conformations of unexpectedly large and small R_g values that would be excluded during the HPLC-SAXS measurement. The six conformations of the best ensemble of R_g values between 44 and 49 Å (Figure 5d) are overlapped respectively with the CORAL model structure (representing an average structure) in Figure 5 to reveal the conformational polydispersity of the **E** form of BSA. Similar SAXS analysis for protein or vesicle conformation polydispersity or flexibility was demonstrated previously.[46,47] The model overlapping in Figure 5 reveals that **IIIB** and **IA** subdomains are relatively more mobile than the other domains (Figure 5b-c), presumably resulted from partial domain melting (particularly in the interfaces of the subdomains as revealed from the local probes detailed in SI). In contrast, the two pivot

DOI: 10.1021/acs.jpclett.6b02722
J. Phys. Chem. Lett. 2017, 8, 470−477

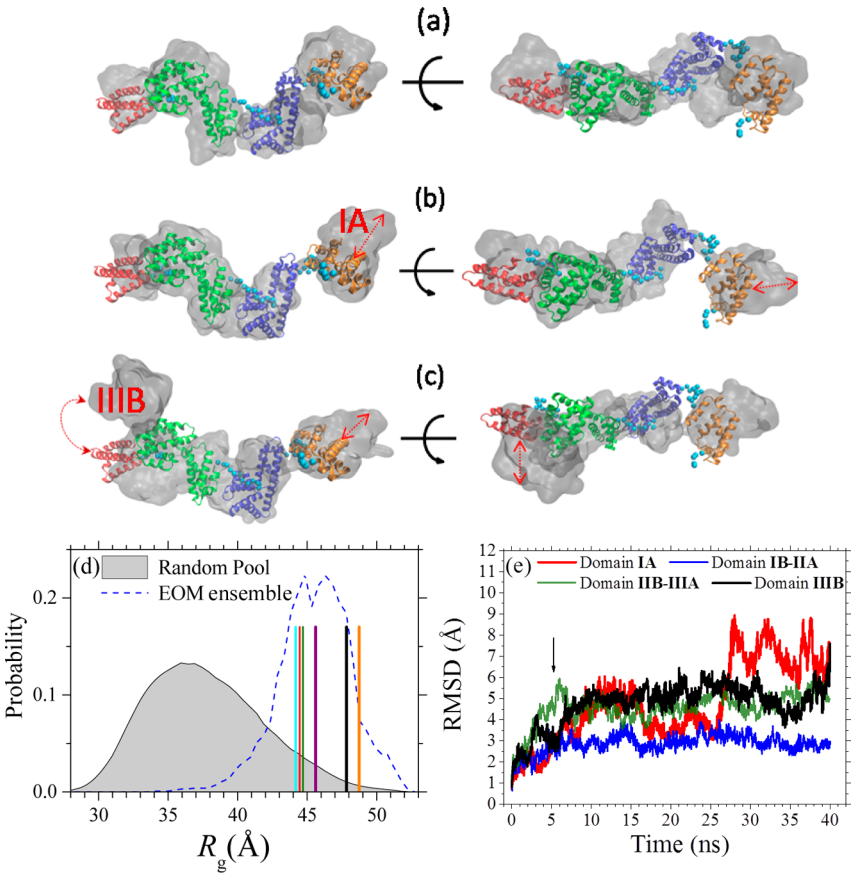

Figure 5. (a–c) Orthogonal views (surface presentations in gray) of the three representative (out of the six in Figure S9) models of the best-fit EOM ensemble for the **E** form of BSA. The models are overlapped with the CORAL model (of colored domains) to illustrate the regional conformation polydispersity, as indicated by the arrows. (d) R_g distribution of the EOM-SAXS selected ensemble (dotted blue profile) centered at ~46 Å in the higher R_g regime of the random pool (gray filled area). The six models that constitute the best-fit ensemble are marked by the vertical bars of equal probability. (e) Evolution of the RMSD for the four EOM-SAXS assigned rigid domains (as marked) during the constant-pH molecular dynamics simulation at pH 2.7. All RMSD values are calculated with respect to the initial coordinates. Larger fluctuations corresponding to the structural features of the **E** form are observed after ~5 ns of the simulation.

units of (**IIIA-IIB**) and (**IIA-IB**) are more steady in the six models as revealed in Figure 5 and Figure S9, suggesting a better core structure stability (or rigidity) of the **E** form. Because the linker (12 amino acids) between **IIA** and **IIB** is flexible, the regional rigidity presumably originates from the attractive domain–domain interactions.

To examine the SAXS-revealed local packing flexibility and rigidity of the molten globule domains in the **E** form, we further conduct constant-pH molecular dynamics simulations with the coupling between protonation and the conformation explicitly taken into account.[48,49] The hence captured dynamic behavior of the **E** form during the 40 ns simulation of the pH-unfolding process, starting from the native BSA conformation at pH 2.7 (300 K), confirms the features revealed by the SAXS EOM models (detailed in Figure S10). Specifically, swing open of subdomain **IIIB** is observed in the earlier stage of the molecular dynamics simulation; after t = 5 ns, pivot opening of the interconnected subdomains of **IB–IIA** and **IIB–IIIA** for the **E** form is observed. Subsequently, the pivot domains remain stable with relatively small root-mean-squared distance (RMSD), as shown in Figure 5e. In contrast, volatile trajectories for subdomains **IA** and **IIIB** continue along the rest of the 40 ns simulation path, resulting in constant mobile subdomains with oscillatory RMSD values (Figure 5e), as suggested by the EOM-SAXS analysis.

In summary, using scanning SAXS along the elution path of HPLC along with molecular dynamics simulation, we have elucidated the long-postulated packing structures of the three molten globule domains of BSA in acid-induced unfolding. The multistep changes of the packing structure observed are mapped onto a three-group unfolding process, on the basis of an unconventional view of protein folding–unfolding formulated with a modified Ising model. Collective changes in the packing structure of the three molten globule domains result in a cleft in the pivot domain interfaces followed by a pivot opening of the central interconnected rigid domains of BSA, characterizing the $\mathbf{I}_1$–$\mathbf{I}_2$ and $\mathbf{I}_2$–**E** transitions. These feature transitions together with the molecular dynamics simulation details provide hints in incorporating or releasing small molecules for biomedical or industrial applications. Most importantly, the observed evolution of the protein conformation strongly supports the previous simulation studies;[7,8] all suggest that collective motions of the molten globule domains, in addition to main-chain or side-chain interactions with the denaturant, profoundly influence the folding–unfolding pathway of a multidomain protein.

DOI: 10.1021/acs.jpclett.6b02722
J. Phys. Chem. Lett. 2017, 8, 470–477

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b02722.

Materials and experimental methods; SAXS data analysis; UV−Vis data; and detailed SAXS parameters (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partially funded by the Minister of Science and Technology, Taiwan (MOST 104-2627-M-213).

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