Protein folding by a quasi-static-like process: A first-order state transition

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In this paper we report that quasi-static-like processes, in which stable intermediates were introduced carefully and deliberately, may be used to reversibly unfold and refold purified native porcine growth hormone. Through circular dichroism (CD) and dynamic light scattering (DLS), we were able to study the secondary structure conformational changes, tertiary structure thermal stabilities, and the particle size distributions of both the intermediates and the final folded product. The CD data showed that the secondary structure was restored in the initial folding stage, whereas the tertiary structure within the protein was restored one step before the last folding stage, as elucidated by thermal stability experiments. DLS analysis suggested that the average hydrodynamic radii of the folding intermediates shrunk to nativelike size immediately after the first folding stage. Our data suggested that the denaturant-containing protein folding reaction is a first-order-like state transition process. This quasi-static-like process may be useful in the prevention of aggregate formation in protein purification and thus can be used in protein engineering to improve the overall yield from harvesting proteins.

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I. INTRODUCTION

Recently, a generalized protein folding model was proposed in which the folding of proteins was found to be consistent with a two-state or two-stage model through a study of the kinetics of hydrogen exchanges of amide with denaturant-containing solutions [1,2]. The basic concept of the two states of protein proposed includes a folded (native) and an unfolded state (denatured) [3], where the definition of ''state'' can be described as a region of configuration space, usually the neighborhood of a potential minimum [2]. The structural changes that occur when a protein unfolds and then refolds may be associated with the alteration of solution conditions according to the energy landscape shift in a kinetic model [1,4,5]. However, when the environment of an unfolded protein under denaturant is changed rapidly to an environment which will cause folding, such as the stop-flow measurement, the protein may be trapped in a subglobal minimal state, generating a ''burst phase'' (BP) intermediate [3] before settling into the final form. Within this ''burst phase'' the conformation of protein is in a partially folded form, which containing some secondary structure [3]. The energy state of this ''burst phase'' intermediate is in the subglobal minimal state, which protein may be trapped during folding processed [2]. That is to say that the folding pathway is likely to be three state, $U \Leftrightarrow \mathrm{BP} \Leftrightarrow N$, instead of two state, $U \Leftrightarrow N$. This BP intermediate may, therefore, play an important role in protein aggregation originating from some form of intermolecular interactions, as observed by Nawrocki *et al.* [6]. The protein aggregation may be caused by the abrupt change of its solution environment. However, if the environment is changed gradually with respect to energy levels, the aggregation of protein might be prevented. To demonstrate this concept, we designed a stepwise quasi-static-like folding process. The folding process is slowed [4,5] by diffusion-controlled stepwise dialysis to alter the folding potentials of the intermediate states (M_i). Furthermore, if the intermediates are stable enough to undergo physical study, it may lead to a better understanding of the structures of the intermediates and the folding processes.

The folding of a protein may be a spontaneous process and independent of its folding path [7], or, alternatively, it may follow a specific pathway *in vivo* [8]. However, in an artificial environment (*in vitro*), both dissolved and precipitated forms of protein may be observed in most high-concentration cases (concentration >0.1–0.3 mg/ml) of folding processes [9]. Combining both *in vivo* and *in vitro* observations may indicate that the same reaction may follow two different paths. Since protein folding has been recognized as a two-state transition model [1–3], this variant folding processes may follow a first-order state transition manner. A first-order transition manner is a transition with a finite transition boundary on a reaction phase diagram. The state transition is discontinuous when the reaction parameters are under the critical point (transition boundary), and the reaction state will continuously change when the parameters are higher than the critical point [10]. Whether the folding of proteins belongs to phase transition or not is still undefined. If the folding transition is following the first-order transition model, two reaction phenomena should be observed during the processes. First, the two states coexisting should be marked by the reactions crossing the state transition boundary. Second, the continuous change is marked by the reaction over the transition boundary.

In this paper, we illustrate a stepwise thermal equilibrium dialysis (TED) approach on the folding pathway of porcine growth hormone (PGH). Native PGH has a four-helix bundle with two disulfide bonds [11] in the structure. The interconvertibility between the folded and unfolded states of PGH

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TABLE I. Compositions of five folding buffers.

Folding buffer	Tris base (mM)	*p*H	Urea (M)	DTT (mM)	Mannitol (%)	Pefabloc. (μM)
1	10	11	2	0.1	0.1	0.5
2	10	11	1	0.1	0.1	0.5
3	10	11		0.1	0.1	0.5
4	10	8.8		0.1	0.1	0.5
5 (native buffer)	10	8.8		0.1		0.5

provides an ideal model for quasi-static-like folding studies. Urea and mannitol are used as steric blocker, which blocks or retards both inter-or/and intra-molecular interactions, and chemical chaperonin, respectively, as reported in a previous study [12].

We conducted circular dichroism (CD) [13–15] and dynamic light scattering (DLS) techniques [16,17] to determine the structures (both secondary and tertiary) and the size distribution of the intermediates by their hydrodynamic radii in each quasi-static-like stage.

Both the quasi-static-like folding process and the burst phase process products fit the first-order transition model. Therefore, we propose that the denaturant-containing folding process is a first-order-like transition. This paper also delineates an alternative method for folding proteins from the denatured aggregation (i.e., inclusion body, where the organism for bacteria storage dysfunction proteins) in quantities without loss through precipitation.

II. MATERIALS AND METHODS

A. Materials and buffers

Native PGH was purchased from Sigma Ltd. (St. Louis, MO). All other chemicals were obtained from Merck Ltd. (Rahway, NJ). The denaturing/unfolding buffer contained 4.5 M urea with 10 mM Tris base (buffer salt), 0.1 M dithiothreitol (DTT) (antioxidation reagent and reduction reagent to break disulfide bonds of protein in high concentrations), 0.1% mannitol (chemical chaperonin), and 0.5 mM Pefabloc (protease inhibitor). There were five folding buffers employed in this study. Their compositions are summarized in Table I.

Both concentrated urea and hydroxyl ion (from NaOH) broke the hydrogen bonds within the protein and changed the protein's conformation. However, urea broke hydrogen bonds by interacting with protein residues directly, but hydroxyl ion alternated the surface charges of protein's solvent accessible area and changed the conformation of the protein indirectly.

B. Folding of PGH by quasi-static-like thermal equilibrium dialysis

The unfolded PGH (U) was obtained by treating it with denaturing/unfolding buffer to make it 10 mg/ml in concentration. The disulfide bonds in PGH are in the reduced form at this stage. This solution was left at room temperature for one hour and then was centrifuged at 4000 g for 30 min to remove the undissolved residue. The folding processes were accomplished by two methods. One of them was a direct dilution of unfolded PGH into a 100 fold native buffer, mixed well at ambient temperature and waited for 10 min until the reaction equilibrium reached. The diluted protein solution was centrifuged at 12 000 g for 30 sec to separate the precipitation portion and soluble portion of protein. The concentration of folded protein was determined by measurement of the absorption at 277 nm by an ultraviolet (UV) spectrophotometer. The extinction coefficient of growth hormone is 700 $g^{-1}\,cm^{-1}\,ml$ [12].

The other is described below as a quasi-static-like procedure. It involves five consecutive TED steps. Each of the folding intermediates is dialyzed against a particular folding buffer at 4 °C.

Step 1. The unfolded PGH (U) in denature/unfolding buffer was dialyzed against folding buffer 1 for 72 h to dilute the urea concentration to 2 M (This produces intermediate 1 or M_1).

Step 2. M_2 was obtained by dialyzing M_1 against folding buffer 2 for 24 h to dilute urea concentration to 1 M.

Step 3. M_3, an intermediate without denaturant (urea) in solution, was then obtained by dialyzing M_2 against folding buffer 3 for 24 h.

Step 4. M_3 was further dialyzed against folding buffer 4 for 24 h, and the pH changed from 11 to 8.8 to produce M_4.

Step 5. Finally, the chemical chaperonin mannitol was removed by dialyzing M_4 against native buffer for 8 h to yield M_5.

C. Theoretical bases of dynamic light scattering

The dynamic light scattering method was introduced to measure the radius (R_H) of the protein solute in terms of the diffusion coefficient (D) according to the following Stokes-Einstein relation [17]:

$$D = \frac{kT}{6\pi\eta R_H}, \tag{1}$$

where k, T, and η are Planck constant, temperature in kelvin, and viscosity of the solution, respectively. The D can be derived from the autocorrelation function (ACF) as $C(\Delta t)$, with $C(\Delta t)$ defined as the running sum of products of the intensity $I(t)$, measured at time t, and the intensity $I(t-\Delta t)$, measured previously for many different values of Δt, which are obtained by measuring the time average of the random scattered signal in the DSL experiment

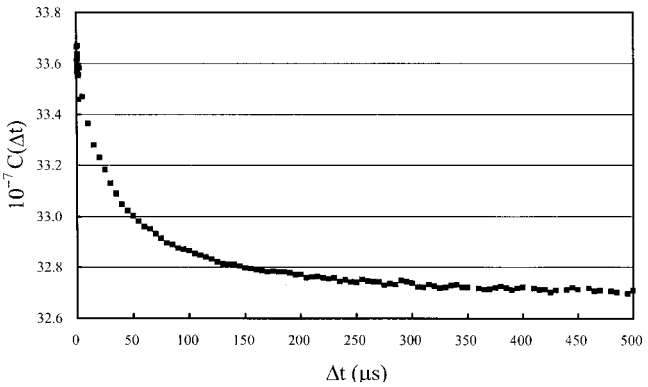

FIG. 1. Dynamic light scattering (DLS) autocorrelation function (ACF) profile of folded PGH. $C(\Delta t)$ denotes the time correlation function as described in Eq. (2). Δt denotes the different time measurement. Details are described in Sec. II C.

$$C(\Delta t)=\langle I(t)\cdot I(t-\Delta t)\rangle. \tag{2}$$

One of the ACF profiles is shown in Fig. 1. The time range was 500 μs. The obtained $C(\Delta t)$ is a function of D [17],

$$C(\Delta t)=A\left\{\sum_i f_i e^{(-D_i Q^2 \Delta t)}\right\}^2+B, \tag{3}$$

where A is an instrument constant, B is the baseline of ACF, and D_i and f_i are the diffusion coefficient and the particle size weighting function of ith particle size, respectively. Furthermore, the ''scattering wave vector'' Q is defined as

$$Q=\left(\frac{4\pi n}{\lambda}\right)\sin\left(\frac{\theta}{2}\right), \tag{4}$$

where n is the reflective index of the suspension solvent, and λ and θ are the laser wavelength and the scattering angle, respectively.

Therefore, the radius (R_H) of each intermediate, including the unfolded and native PGH, can be determined by Eqs. (1)–(4) by measuring of $C(\Delta t)$ (Fig. 1).

D. Analysis of size distribution of refolding PGH in steps 1–5 by dynamic light scattering

Before the measurement, PGH in each dialyzing step had to be diluted to 0.2 mg/mL. The light-scattering apparatus was equipped with an argon ion laser light source (4880 Å) (Ion Laser Tech., UT). The scattered light was collected at 90° by a goniometer from Brookhaven Instruments Corp. (BIC, Holtsville, NY). The chamber temperature was controlled by a water circulator. The ACF was computed using a digital correlator (BI9000), then analyzed by the nonnegatively constrained least-squares (NNLS) method [18] based on the theory provided by BIC. The instrument performance was calibrated by measuring the DLS of standard suspensions of polystyrene bead ($R_H=68$ nm) (Polysciences, Warrington, PA). All experiments were conducted at 20 °C.

E. Analysis of protein secondary conformation

CD spectra in the UV region (320–200 nm) of U, M_{1-5}, and N were recorded on a Jasco J 720C spectropolarimeter at 20 °C. A 0.1 cm light path cuvette was used to reduce the light scattering of the solution. Data are expressed in molar ellipticity (deg cm^2 dmole^{-1}), calculated based on molecular weight of 22.6 kD for PGH. The measured CD spectrum of each individual sample was diluted with the same folding buffer of each stage. The solvent contribution on CD was subtracted from each spectrum. The component secondary structures, i.e., α helix, distorted α helix, β sheet, distorted β sheet, turns, and unordered, were analyzed by the singular value decomposition algorithm provided by the Selcon 3 program [19] (Fig. 3).

The formation of tertiary structures of folded PGH was monitored by the CD ellipticity profile at 222 nm versus temperature from 10 to 90 °C. The thermal stability of the tertiary structure of PGH was determined by the reflection point of the melting curve [14].

III. RESULTS AND DISCUSSION

A. Quasi-static-like TED folding processes avoid diffusion-limited aggregation

As described by the energy landscape model [1], a denaturant may change the reaction potential of protein folding. Therefore, systematically changing the urea concentration and the *p*H value in the folding solution may change the folding pathway of the protein. However, rapid dilution of a high concentration of unfolded PGH (U) by the native buffer caused precipitation, possibly due to protein trapping in a subglobal state or a burst phase [20,21]. Similar effects have been observed on cytochrome *c*, RNase *H*, and *T*4 lysozyme [22–26]. These aggregations have been assumed to be a diffusion-limited process, *in vitro* [27]. Therefore, it is important to change the solution conditions as slowly as possible to avoid the protein being trapped in the burst phase and subsequently forming aggregates [9].

During the dialysis, the flow rate **j** of solutes (urea and/or hydroxyl ion in this study) is regulated by its diffusion coefficient (D) and the concentration gradient (∇n), i.e., $j\propto -D\nabla n$. In order to approach a quasi-static-like process, **j** has to be reasonably small. In protein denaturation by urea, the optical molar ellipticity of the protein changed a great deal of 2 M urea and reached a plateau around 1 M urea [28]. Therefore, to regulate the diffusion rate of urea interacting with the proteins, a three-step folding TED process against urea (4.5, 2, and 1 M) and one against hydroxyl ion (changing *p*H from 11 to 8.8) were used for mimicking a quasi-static process. The time scale of protein folding is around a submicrosecond to millisecond [4,5] *in vitro*. In this study, we changed the folding buffers to retard the folding processes 152 h. Therefore, the folding process of our experiment is about 10^8 to 10^{11} times slower than the nativelike folding processes [4,5], and we call this approach a quasi-static-like folding process.

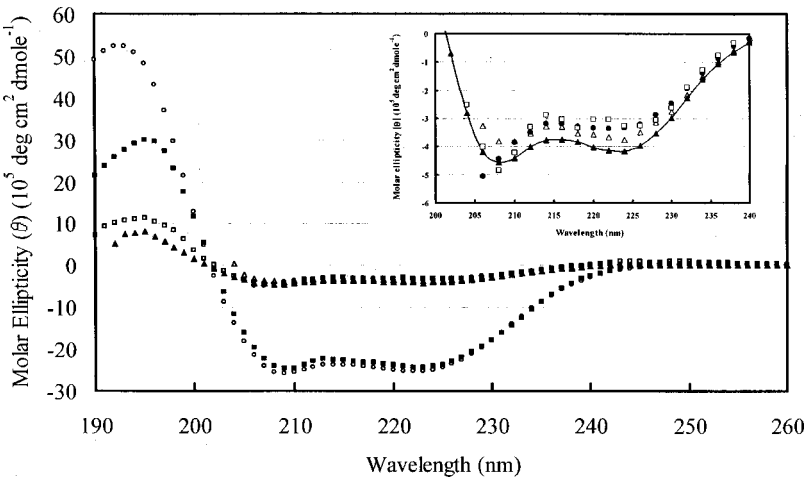

FIG. 2. CD profiles of PGH at 20 °C. The symbols open circle, filled circle, open triangle, filled triangle, open square, and filled square represent native form (N), M_1, M_2, M_3, M_4, and M_5, respectively. The CD was taken at 0.2 nm a datum point but only 1 nm a datum point is shown for clarity. The inset is the enlargement of CD 200 to 240 nm of M_{1-4}. The similarity of CD curves of M_{1-4} was illustrated by the smoothness of M_3.

B. Secondary structures of stable folding intermediates of PGH (M_{1-5})

In the unfolding buffer, the CD of U showed no meaningful spectral features in wavelengths shorter than 210 nm (data not shown). Therefore, the unfolded PGH had no analyzable structures [19]. Their optical characteristics were restored after the 72 hr TED process during which the urea concentration changed gradually from 4.5 to 2 M in the first folding step (M_1). Its secondary structures can be analyzed as shown in Fig. 3. It contains 58% α helix, 22% distorted α helix, 5% turn, and 15% unordered, and no β sheet was detected. This composition is identical to that of the native PGH (Fig. 3). Subsequent quasi-static-like studies (see Sec. II B) showed that the CD profiles of $M_{2,3,4}$ are strikingly similar to that of M_1 (Fig. 2 and inset) which indicated that the secondary structures of these intermediates are the same as M_1. The CD intensities increased when the chemical chaperonin (mannitol) was removed from solution as M_4 became M_5. The difference of CD intensities between M_4 and M_5 (and N) may be caused by mannitol removal. Mannitol, like other poly-ols, protects side chains of proteins from being unexpectedly modified by a highly reductive/oxidative environment. However, the feature of the CD curve profile still resembles that of M_4 (Fig. 2). Therefore, the composition of the secondary structure has not changed since the M_1 stage (Fig. 3). This is further evidence to show that the secondary structures of PGH were restored as the urea concentration was decreased to 2 M.

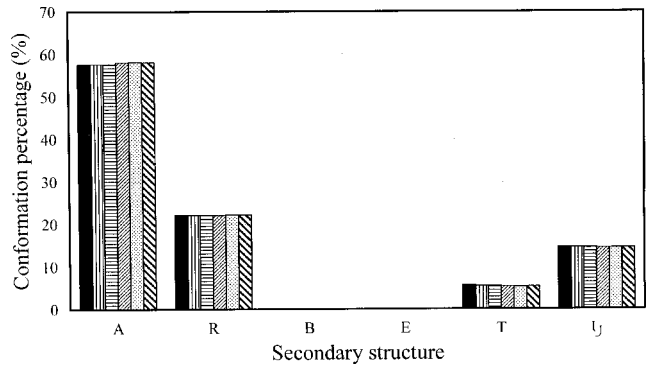

FIG. 3. CD profiles analysis of growth hormones by Selcon 3 [14]. The solid, vertical line, horizontal line, slant (from low left to high right), shaded, and slant (from high left to low right) represent N, M_5, M_4, M_3, M_2, and M_1, respectively. In addition, the secondary structure is represented by the following: *A*, regular α helix; *R*, distorted α helix; *B*, regular β strand; *D*, distorted β strand; *T*, turns; and *U*, unordered. The α-helical and β-sheet structures were split into regular and distorted classes by considering four residues per helix and two residues per β strand distorted [19].

C. Tertiary structure of stable folding intermediates of PGH (M_{1-5})

The formation of the tertiary structure of PGH was monitored by the melting curves at 222 nm of CD. The unfolded U has no reflection point on the melting curve. Thus, it has no cooperative transitions in structure, either. Similarly, there were no thermal transitions observed for $M_{1,2,3}$, which indicated that no tertiary structures were formed in these intermediates. On the contrary, there were melting points observed at 73 and 74 °C in M_4 and M_5, respectively, which were the same as, or close to, the melting point of native porcine PGH (74 °C) under the same conditions (Table II). This means that the tertiary structure was about to be restored when the *p*H was dropped from 11 to near neutral (8.8). The melting point and the CD spectra (except at very short wavelengths, Fig. 2) of M_5 are identical to those of the native PGH. The tertiary structure of protein can be indirectly monitored by the thermal denaturation CD profiles. The ellipticity attenuation by thermal denaturation forming a sigmoidal curve of CD profile at 222 nm indicated that the helical to coil structure transition is cooperative. This cooperation may cause by the interactions between the helix and side chain of tertiary structures [13]. The same or similar Tm

TABLE II. Thermal denaturation melting temperature (Tm) of the intermediates M_4, M_5, and N of porcine growth hormone (PGH) at 222 nm on CD.

PGH	Melting temperature (°C)
M_4	73.3
M_5	74.4
N	74.4

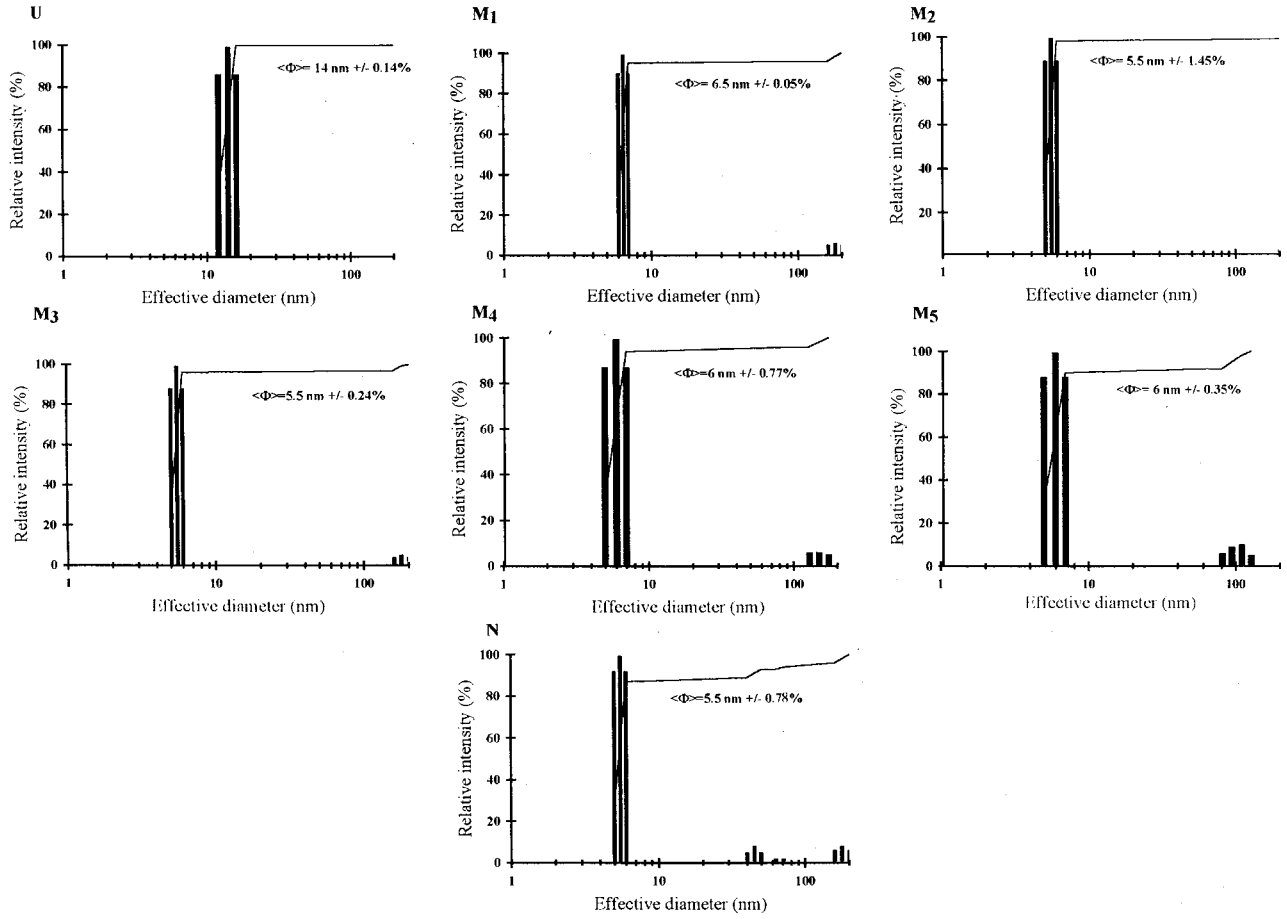

FIG. 4. Particle size distribution of variant stages of U, M_{1-5}, and N of PGH. The solid lines indicate accumulation percentage of particles (see text). (Those portions with particle size larger than 40 nm may be artifact and are negligible.)

value can represent the tertiary structure forming [12,13,15]. We therefore concluded that at M_5 the PGH had been folded back to its native form.

According to the above CD results (spectral characteristics and thermal stability), we found that the helical structure of PGH formed at 2 M of urea concentration. The preliminary tertiary structure of PGH was about to be formed when the *p*H returned to near neutral. Finally, the structure of PGH was totally restored by the removal of mannitol chemical chaperonin.

D. DLS studies on PGH intermediates (M_{1-5})

In solution, dynamic light scattering is one method for measuring particle size distribution (PSD) size in the submicron range [16]. As shown in Fig. 4, the DLS measurements indicated that the sizes of all folding intermediates are close in single distribution. The uniformity of the diameters can be considered as evidence for a quasi-static-like process and as a necessity for preventing precipitation. This also indicated that the TED process reached equilibrium and that the intermediates were in a uniform state rather than a mixture of ''folded'' and ''unfolded'' forms. The effective diameters (R_H) of U, M_{1-5}, and N measured were 14 ($\pm$0.14%), 6.5 ($\pm$0.05%), 5.5 ($\pm$1.45%), 5.5 ($\pm$0.24%), 6 ($\pm$0.77%), 6 ($\pm$0.35%), and 5.5 ($\pm$0.78%) nm, respectively, at 20 °C. The particle size of refolded growth hormone (M_5) is 6 nm and the crystal structure is 5.5 nm [11]. Therefore, the hydration layer is about 0.25 nm [$(6-5.5)/2$]. This distance is equal to a single layer of water. These measurements were calibrated against reference polystyrene beads with $R_H=68$ nm. However, the measuring of standard suspensions of polystyrene beads have 72 ($\pm$0.14%) nm diameters. This indicated the error range of the instrument performance is around 6%. Although the compositions of secondary structure among all intermediates (M_{1-5}) are the same, their aggregation broke rapidly as the urea was diminished by dialysis and changes in pH. These observations in Figs. 2, 3, and 4 indicated that the stepwise dialysis of urea was necessary in establishing a quasi-static-like process in folding without the formation of any precipitate.

In comparison, the diameter of the human growth hormone that has a structure similar to native PGH, obtained from *x*-ray crystallography, is around 6 nm [11], which is very close to that of M_5 and measured N. Therefore, the unfolded PGH was indeed being folded to its native structure by the quasi-static-like process described above.

E. Protein folding is consistent with both collapse and sequential models

As described in the above section, the R_H fluctuates within the range (from 6.5 to 5.5 nm) of all the intermediates

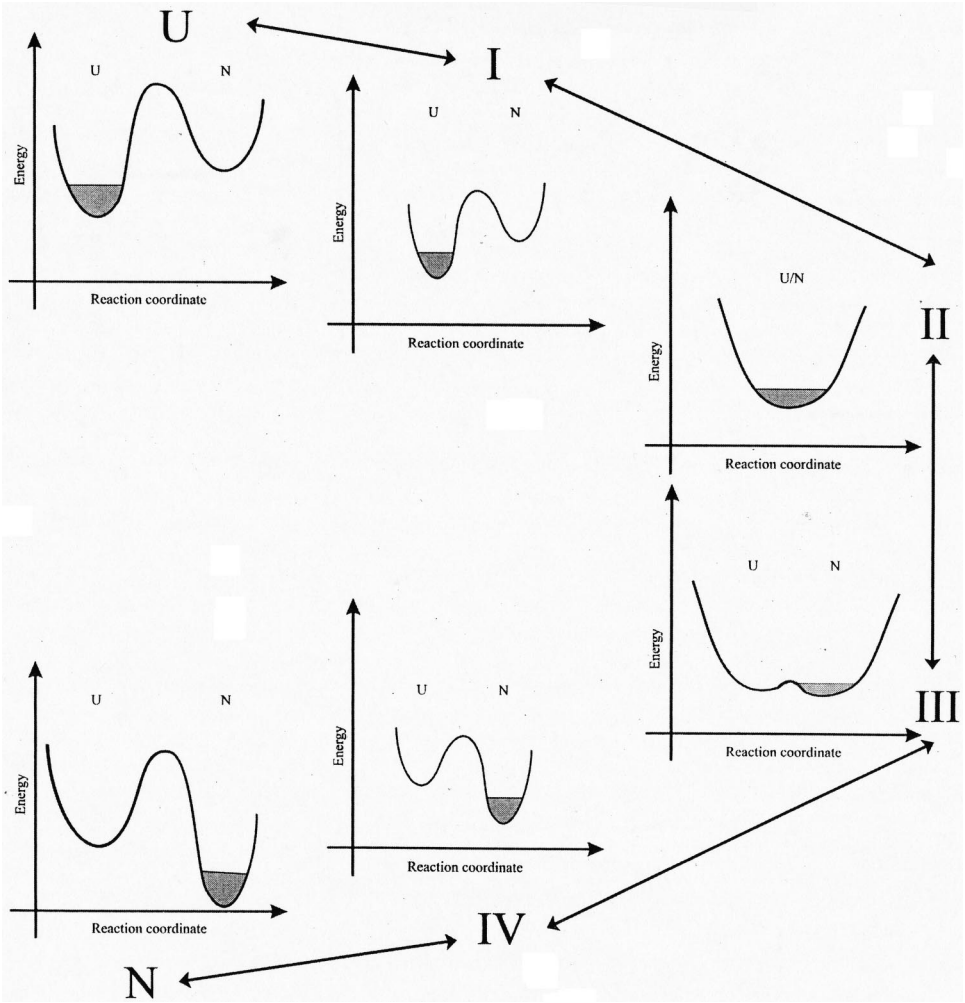

FIG. 5. A cartoon of the possible potential change in the quasi-static-like process. Where *U*, *I*, *II*, *III*, *IV*, and *N* denote the five-folding stage (M_{1-5}) and native form, respectively. The local minimum is gradually changed from the unfolded to the folded state.

and the native form. The phenomena of the proteins' collapse in particle sizes and the formation of secondary structures prior to their tertiary structural interactions are consistent with the collapse model of protein folding [29].

Both CD (Figs. 2 and 3, Table II) and DLS (Fig. 4) showed that the first three intermediates were in neither the unfolded nor the folded state, but they contained secondary helical structures. They may be classified as molten globule-like forms. The formation of secondary conformation may follow the sequential model of Kuwajima and Schmid [30]. Namely, it folds in the following manner: $U \Leftrightarrow M_1 \Leftrightarrow M_2 \Leftrightarrow M_3 \Leftrightarrow M_4 \Leftrightarrow M_5(N)$. The intermediates ($M_i$) differ from burst phase (BP), and they are fairly stable and can be considered as a nativelike state (N') under such conditions.

F. Protein folding process is a first-order-like state transition process

As indicated by Qian and Chan [1], denaturants, such as the hydroxyl ion and urea used in this study, change the potential energy levels of protein folding. The observation of uniform PSD of each of the folding intermediates suggested that they reached equilibrium under each folding solution, as mentioned previously. These equilibrium phenomena indicated that the refolding intermediates (M_1 to M_4) tended to remain at the lowest-energy state locally during the process, until the native form (N) was reached. Based on our observations, we propose the existence of a protein folding process in which the potential changes continuously in a quasi-static-like TED manner, as indicated in Fig. 5. The unfolded protein (U) is at equilibrium in the folding solutions. The intermediates are always located at the lowest-energy state under certain solution conditions. For this reason, we could not observe a two-state transition during the process. This postulation is similar to the description of a first-order gas-liquid transition under a specific path, which detours from the critical point and can go continuously from a gas to a liquid state without ever going through a phase transition [10].

As described in Sec. II B when diluting an unfolded protein rapidly in 100 fold into a native buffer, results from UV observation showed that only 10% of the protein is folded in solution, and 90% of the unfolded protein is aggregated and precipitated. The CD profile of this soluble portion is identical with folded PGH (data not shown). In other words, by diluting the completely unfolded protein into a native buffer directly, both an aggregation and a soluble portion can be found. Nawrocki *et al.* [6] results indicated that this aggregation may be caused by ''burst phase'' intermediates. This ''burst phase'' intermediate is a folding intermediate that is

formed during the dead time of stop-flow measurement (~18 ms [3] or 2 ms [6]).

Therefore, based on the two-state transition model of protein folding [2], these irreversible aggregation proteins can be classified as the unfolded state and the folded state (i.e., the soluble portion), respectively. In a review, Misawa and Kumagai have described similar observations for other proteins [9].

In a state of minimal free energy (the equilibrium state), mean-field theory (Ginzburg-Landau theory) states that a first-order transition is a discontinuous one under the critical point [10]. At the critical point, there is an area under which the two states, e.g., *U* and *N*, can coexist in a phase diagram. This phenomenon was observed under the rapid dilution process of protein folding in this study (data not shown). However, a quasi-static-like process can make an alternative pathway and cause continuous changes from the unfolded (*U*) to the folded (*N*) state. In other words, the folding reaction is initiated from unfolded protein by the denaturant which interacted and unfolded protein (denaturant-containing protein) and fold into a native state of protein (without denaturant). Therefore, we propose that, in general, folding of denaturant-containing protein is a first-order-like state transition which is similar to Ptitsyn's prediction [31].

G. Protein may fold with a continuous transition path in a cell

Cells avoid accumulating potentially toxic aggregates by mechanisms including the suppression of aggregate formation by molecular chaperones and the degradation of misfolded proteins by proteasomes [27]. In eukaryotic systems, HSP70 serves as molecular chaperonin, which directly interacts with unfolded proteins, and HSP60-HSP10 complex provides a hydrophobic cave in facilitating protein folding [32]. In prokaryotic systems, GroEL and GroES play roles similar to those of HSP60-HSP10 complex [33]. These direct or indirect interactions with unfolded proteins will change the reaction potential in a way similar to the behavior of urea and *p*H in this study. Moreover, under physiological conditions, proteins such as growth hormones will not aggregate. These observations indicated that chaperonins help the protein fold while preventing the state transition from taking place. Our studies with chemical molecules mimic those effects of molecular chaperonin. In fact, those *in vivo* reactions are more efficient than our *in vitro* studies. Therefore, we predict that those proteins, *in vivo*, which have the ability to fold may follow a continuous path without state transition.

IV. CONCLUSION

Stable protein folding intermediates can be obtained by using quasi-static-like TED processes. The conformations and the particle size distributions of the intermediates can be detected by CD and DLS, respectively. Comparison with discontinuous transition by direct dilution and continuous change by quasi-static-like TED processes suggested that a denaturant-containing protein folding reaction is likely to be a first-order-like state transition process. This implies that a protein can go continuously from an unfolded state to a folded state without ever going through a phase transition. Namely, an unfolded protein that folds back through a stepwise quasi-static-like process, as suggested in this paper, may experience a gradual change in its environment and thus will not be trapped in a ''burst phase'' or form aggregates. This delineates an alternative method for folding a protein from its denatured aggregation (i.e., inclusion body) back to its native form in larger quantities.

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