Long-Lived Conformational Isomerism of Protein Dimers: The Role of the Free Energy of Subunit Association

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ABSTRACT The association of protein subunits to form N-mers ($N \geq 3$) does not follow the dependence on the law of mass action predicted by the classical thermodynamic description used for the equilibrium of association of small molecules. For those anomalous cases, a so-called deterministic model has been previously proposed. The latter model was based on the empirical observation that the dynamics of subunit exchange between protein oligomers can be very slow, leading to the existence of long-lived conformational isomers and to a persistently heterogeneous ensemble of oligomers in solution. Contrary to the expectation for a protein dimer, we have recently shown that the subunit association of triosephosphate isomerase (TIM) could also be described as a deterministic process and that long-lived conformational isomers of TIM could be isolated in solution. Here we show that a), observation of hysteresis in pressure dissociation curves is an additional indicator of deterministic behavior; b), the extent of deviation from the classical thermodynamic behavior correlates with the free-energy change of subunit association; and c), experimental manipulation of the free energy of subunit association through the addition of a subdenaturing concentration of a chaotropic agent restores the concentration dependence of subunit association of TIM. A model that explains these features and its biological relevance is discussed.

INTRODUCTION

The classical description of the energetics and kinetics of protein oligomerization is based on the assumption that association and dissociation are stochastic processes. For this assumption to be valid, a large number of equivalent protein molecules must be in rapid equilibrium between monomeric and oligomeric states. If this were true, the thermodynamic formalism developed for small molecule reactions would be fully applicable to the description of protein association reactions as well. However, early studies have shown that this type of description is completely invalid for large protein oligomers, such as virus capsids, and has limited validity even for oligomers containing three or more subunits (reviewed in Silva and Weber (1)). For those particles, a model assuming long-lived conformational heterogeneity has been proposed that agrees well with the experimental data (2). On the other hand, it has been generally assumed that the stochastic model is valid for protein dimers. However, we have collected data on triosephosphate isomerase (TIM), a dimeric protein, showing that this dimer also exhibits considerable conformational heterogeneity (isomerism) in solution and that its equilibrium of subunit association cannot be adequately described by the stochastic model (3–5).

In this study, we set out to investigate the energetic basis for this ''anomalous'' behavior of TIM. We propose a model which allows the prediction, for any protein dimer, of the extent to which the purely stochastic model of subunit association/dissociation may be considered to be valid. In the following, the basic features of the classical thermodynamic description of protein subunit association are briefly recapitulated and then compared to recent experimental data on the subunit association of TIM.

Stochastic nature of subunit association/dissociation in oligomeric proteins

In a fast, dynamic equilibrium between n protein monomers (M) and an oligomer (A) composed of n subunits:

$$n \cdot M \rightleftarrows A,$$

the macroscopic behavior of the ensemble of oligomers is the result of the stochastic outcome of a large number of microscopic association-dissociation events. The dissociation constant, K, and the corresponding Gibbs free-energy of dissociation, ΔG, are given by

$$K = \frac{[M]^n}{[A]} \quad (1)$$

$$\Delta G = -RT \ln K, \quad (2)$$

and the degree of dissociation, α, is defined as

$$\alpha = \frac{[M]}{[C]}, \quad (3)$$

where $[C]$ is the total protein concentration ($[M] + n[A]$) expressed in monomers. Let us now consider the introduction of a physical or chemical perturbing agent that causes a linear decrease in ΔG (for example, linear dependences of

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ΔG are often assumed for perturbation by hydrostatic pressure (1) or chaotropic agents (6)):

$$\Delta G = \Delta G_0 + m[\text{perturbant}]. \quad (4)$$

In this equation, [perturbant] is the magnitude of applied perturbant (e.g., pressure, concentration of chaotropic agent) and m is a parameter that measures the steepness of ΔG as a function of [perturbant]. For pressure perturbation, m is equivalent to the standard volume change upon subunit dissociation, ΔV, whereas for perturbation by chaotropes m has been shown to reflect the accessible surface area that becomes exposed upon subunit dissociation or unfolding of individual polypeptide chains (7). Combining Eqs. 1–4 gives the dependence of the degree of dissociation on protein concentration $[C]$ and [perturbant]:

$$\frac{\alpha}{1-\alpha} = \frac{K}{n^n[C]^{n-1}} e^{\frac{m[\text{perturbant}]}{RT}}. \quad (5)$$

From Eq. 5, one can derive an expression that allows prediction of the shift in midpoint of the dissociation curve ($\Delta[\text{perturbant}]_{1/2}$) when protein concentration is changed from $[C_1]$ to $[C_2]$ (1,7):

$$\Delta[\text{perturbant}]_{\frac{1}{2}} = (n-1)\frac{RT}{m}\ln\frac{[C_2]}{[C_1]}. \quad (6)$$

As expected from the law of mass action, the dissociation curve is predictably displaced when protein concentration is changed. It is noteworthy that the predicted displacement is proportional to n (the number of subunits in the oligomer). Hence, the dissociation curves would be expected to be increasingly sensitive to changes in $[C]$ for higher order oligomers.

The m value, which gives a measure of the cooperativity of the transition, can be obtained from two different types of experiments. First, it can be determined by fitting Eq. 5 to a single degree of dissociation (α) versus [perturbant] curve (from which m_p is obtained, in which the subscript p refers to perturbant). Alternatively, it can be calculated from the measured displacement of the dissociation curve that takes place when the concentration of protein is varied, using Eq. 6 (from which m_c is obtained, in which the subscript c refers to protein concentration). The extent to which these two independently calculated m values agree provides a sensitive index of how closely the equilibrium of subunit association reflects the stochastic outcome of microscopic association-dissociation events. For truly stochastic systems, the γ value (defined as $\gamma \equiv m_p/m_c$) is predicted to be unity (1).

Experimental observation of deviations from the stochastic behavior

For most protein dimers that have been investigated by pressure-induced perturbation of subunit dissociation, Eq. 6 holds to a fair extent and γ was indeed found to be close to unity (reviewed in Silva and Weber (1)). On the other hand, relative to the values predicted by Eq. 6, trimers and tetramers exhibit significantly reduced displacements of the dissociation curves when protein concentration is changed, resulting in $\gamma < 1$ (1,8). Significantly, for large protein multimers, such as virus particles and giant extracellular hemoglobins, the dependence of the transition curve on protein concentration is abolished altogether (i.e., $\gamma = 0$) (9–13). As noted above, this is in sharp contrast with the predictions based on Eq. 6. Thus, in those cases the underlying assumption that the macroscopic behavior of the ensemble of oligomers results from the stochastics of a large number of microscopic subunit association-dissociation events cannot be taken as true any longer.

Initial insight into this apparent conundrum was provided by Erijman and Weber (2). For tetramers, they compared the rate of subunit exchange (i.e., the kinetics of dissociation of a given subunit from one oligomer followed by its association to a different subunit to form another oligomer) to the rate of dissociation after a pressure jump that elicited 50% dissociation of all oligomers in the ensemble. According to the stochastic model, these two rates should be similar. However, the experimentally observed rate of dissociation was much faster than the kinetics of subunit exchange between oligomers. Thus, they concluded that there was a fraction of the tetramers in the ensemble that was persistently more stable and resistant to the dissociating effect of pressure. They attributed this energetic heterogeneity in the ensemble to a persistent (i.e., long-lived relative to the experimental timescale) conformational heterogeneity of the protein oligomers. According to that model, when pressure is applied to the system the fate of an individual oligomer is predetermined by its conformational state at the beginning of the experiment. Conceivably, sufficiently high energy barriers of interconversion (ΔG_{INT}) exist between such different conformational states, preventing conformational exchange during the time of the experiment (typically of several hours). As a result, those different conformational states (conformational isomers) should be treated as distinct chemical species that are not in fast, dynamic equilibrium with each other. Consequently, at any given time the macroscopic behavior of the oligomer ensemble is not given by the stochastic averaging of a large number of dynamic dissociation-association events, but rather is predetermined by the distribution of conformational states of individual oligomers. Erijman and Weber (2) described this behavior as deterministic, in analogy with the behavior of macroscopic bodies and as opposed to the stochastic behavior described by Eqs. 1–6.

In a deterministic process, the shape of the subunit dissociation curve (as defined by the values of $[\text{perturbant}]_{1/2}$ and m) is predetermined by the initial distribution of conformational isomers, which is independent of protein concentration. This explains the lack of, or significantly reduced, protein concentration dependence experimentally observed

in the dissociation curves of some protein oligomers. The γ value described above is a convenient index of whether dissociation follows the stochastic behavior ($\gamma = 1$) or a deterministic transition ($\gamma = 0$). Using this criterion, it was originally concluded that dimers in general exhibit stochastic behavior, whereas multimers such as erythrocruorin, hemocyanin, and viruses feature deterministic subunit association, with tetramers and trimers showing intermediate behavior (1).

Another interesting observation in the pressure dissociation of oligomeric proteins is that, in many cases, pressure dissociation curves measured upon increasing pressure do not match the reassociation curves obtained on decompression after full dissociation (1). This hysteresis can be quantified by taking the difference at midpoint ($\Delta p_{1/2}$) of the curves obtained upon compression and decompression. It is important to note that the stochastic approach (Eqs. 1–6) predicts a unique outcome of the degree of dissociation, α, under a given set of thermodynamic conditions, regardless of the history of the sample. Thus, the observation of hysteresis represents a clear deviation from that model. The dependence of α on sample history indicates that either the compression or decompression curve (or both) does (do) not correspond to the state of lowest Gibbs free energy. Hysteresis indicates that the monomers and multimers present in solution exhibit cycles of association/dissociation that are slow relative to the timescale of the experiment. Thus, similar causes can be identified for both hysteresis and deterministic behavior, namely the existence of high free-energy barriers that prevent the establishment of a true dynamic equilibrium between the species in solution.

The three phenomena described above (deviations from the law of mass action, long-lived energetic/conformational heterogeneity or isomerism, and hysteresis) define the limitations of the stochastic description of protein association. Intriguingly, all three phenomena were reported by us in the pressure dissociation/unfolding of dimeric TIM (3). The results obtained on this dimer suggest that a further reevaluation of the boundaries of validity of stochastic description of protein association equilibria is in order.

The relationship between Gibbs free-energy change and anomalous subunit dissociation of protein dimers

Fig. 1 shows plots of γ-values (*panel A*) and hysteresis (expressed as $\Delta p_{1/2}$, *panel B*) as a function of the Gibbs free-energy of dissociation for nine dimers for which pressure dissociation data are available in the literature. As discussed above, the dissociation of TIM by pressure does not conform to the predictions of the stochastic model; hence, it was not possible to determine the free-energy of dissociation by fitting Eq. 5 to pressure data (3). Instead, the free-energy change was obtained from experiments in which subunit dissociation/unfolding were induced by incubation of TIM samples for 3 days (to ensure complete equilibration) in the presence of increasing concentrations of guanidine hydrochloride (4). Under these conditions, the equilibrium model derived from stochastics was found to adequately describe the experimental data, allowing calculation of ΔG_{diss}.

At free-energy values close to 40 kJ/mol, we find four dimers (R17 phage capsid protein dimer, enolase, plasma membrane Ca^{2+}-ATPase, and arc repressor) that exhibit no hysteresis and little or no deviation from the law of mass action, as indicated by their γ-values close to unity (Fig. 1, *A* and *B*). Thus, stochastic description of the subunit association/dissociation of those dimers is completely successful. In a transition region around 50–60 kJ/mol, four dimers (apo and holo tryptophan synthase, rubisco, and hexokinase) exhibit noticeable deviations from the stochastic model, revealed by γ-values down to $\sim$0.6 and hysteresis of up to 0.8 kbar. This trend culminates in the last dimer, TIM, with $\Delta G = 70$ kJ/mol, $\gamma = 0$, and hysteresis = 1.54 kbar. Pressure dissociation of TIM is a completely deterministic process as judged by these parameters, to an extent only previously

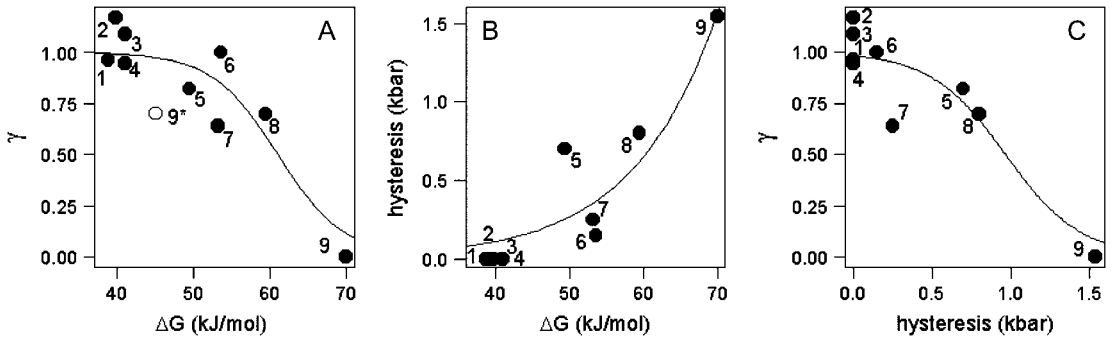

FIGURE 1 Correlations between Gibbs free-energy changes of dissociation (ΔG), hysteresis of pressure dissociation, and γ-values of nine dimers described in the literature. Data are shown for R17 capsid protein dimer (1, (12)), enolase (2, (14)), Ca^{2+}-ATPase (3, (15)), arc repressor (4, (16)), tryptophan synthase apoprotein (5, (17)) and holoprotein (6, (17)), rubisco (7, (18)), hexokinase (8, (19)), and triosephospate isomerase (9, (3,4)). The open circle in panel *A* represents data obtained from pressure dissociation of TIM in the presence of 0.4 M GdnHCl (see Fig. 3 and text for details). Lines are model-free polynomial fits that take into account the restrictions that $0 \leq \gamma \leq 1$ and hysteresis ≥ 0.

described for multimers of dozens of subunits (1). Fig. 1 thus shows the full range of behavior from stochastic to deterministic subunit association in dimers.

As mentioned above, hysteresis and deviations from the law of mass action are both thought to be caused by high activation barriers for subunit exchange between oligomers in solution. The relation between activation free-energy ($\Delta G^{\ddagger}$) and the total free-energy change (ΔG) of a process may be described by linear free-energy relationships such as the Brønsted equation ($\partial\Delta G^{\ddagger}/\partial\Delta G = \beta$), i.e., the activation free-energy barrier for a given reaction is proportional to the equilibrium free-energy change for that reaction. Indeed, for protein-folding reactions it has been shown that $\Delta G^{\ddagger}$ and ΔG correlate when protein stability is changed by mutagenesis or by addition of chaotropic agents, though not necessarily in a rigorously linear way (20). Based on this, we propose the model depicted in Fig. 2 to explain the correlations observed in Fig. 1. From the Brønsted equation, it can be expected that the activation free-energy barrier responsible for hysteresis and for deviation from the law of mass action is correlated to the equilibrium ΔG of dissociation. Above certain ΔG values (40–50 kJ/mol), the activation barriers ($\Delta G^{\ddagger}$) become too high and the subunit exchange between oligomers becomes too slow to allow fast, stochastic equilibrium. This results in γ-values lower than 1 and hysteresis. As expected from this model, hysteresis also correlates directly with γ (Fig. 1 *C*), further suggesting a common energetic basis for hysteresis and for deviations from the law of mass action.

Experimental manipulation of the free energy of subunit association restores protein concentration dependence in the subunit dissociation of TIM

If indeed this type of anomalous behavior arises from a high activation barrier for subunit dissociation/exchange (which in turn reflects a large free energy of subunit dissociation), then it should be possible to modify the behavior of a protein dimer from deterministic to stochastic by experimental manipulation of the free energy of dissociation. To test this hypothesis, we have examined the pressure dissociation of TIM in the absence or in the presence of a subdenaturing concentration of GdnHCl. Confirming our previous results (3), Fig. 3 (*solid symbols*) shows that pressure dissociation/unfolding of TIM in the absence of GdnHCl is completely independent of protein concentration. We then carried out pressure dissociation of TIM in the presence of 0.4 M GdnHCl. At this concentration, GdnHCl does not cause unfolding of TIM (4,5), but produces a change in free energy of subunit dissociation, as described by Eq. 4. The change in free energy of dissociation induced by GdnHCl is clearly demonstrated by the shift of the pressure dissociation/unfolding curves to lower pressures in the presence of GdnHCl (Fig. 3, *open symbols*). Using Eq. 4 and the values of ΔG_o (70 kJ mol^{-1}) and m (63 kJ mol^{-1}M^{-1}) from our previous work (4), one can calculate that addition of 0.4 M GdnHCl causes a reduction in the free energy of dissociation of TIM dimers to ~45 kJ/mol. According to Fig. 1, this should lead to a transition from deterministic to stochastic behavior of subunit dissociation. Indeed, Fig. 3 (*open symbols*) shows that, in the presence of 0.4 M GdnHCl, the dissociation of dimeric TIM by pressure became dependent on protein concentration. For the two protein concentrations used in the experiment shown in Fig. 3, a difference in pressure at midpoint transition ($\Delta p_{1/2}$)

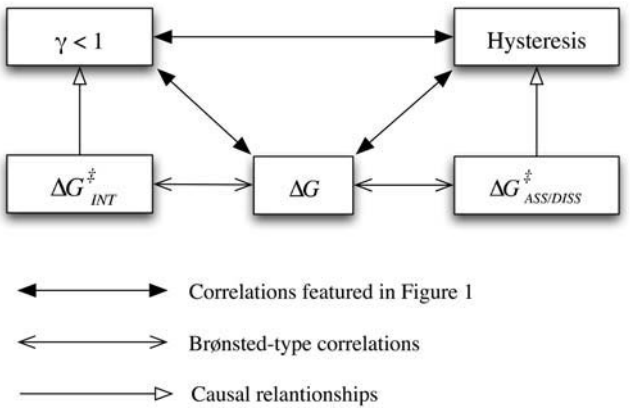

FIGURE 2 Proposed relationships between Gibbs free-energy change (ΔG), γ, hysteresis, and activation free-energy barriers for interconversion between conformational states ($\Delta G^{\ddagger}_{INT}$) or for association/dissociation ($\Delta G^{\ddagger}_{ASS/DISS}$).

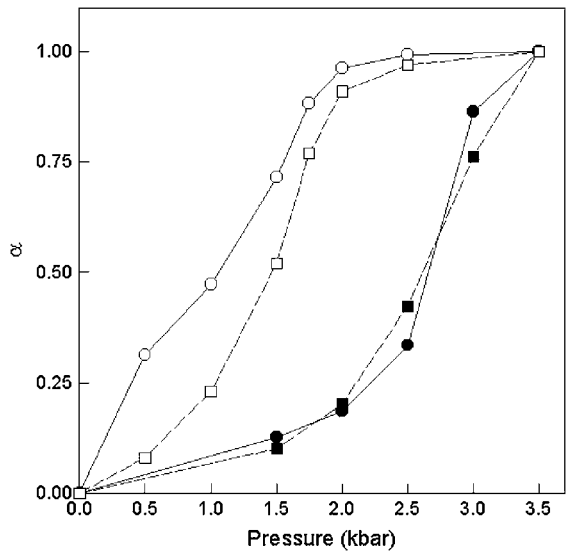

FIGURE 3 Pressure-induced subunit dissociation/unfolding of TIM in the absence or in the presence of a subdenaturing concentration of GdnHCl. Pressure experiments were carried out as previously described, and the degree of dissociation/unfolding (α) of TIM as a function of pressure was calculated from shifts in intrinsic fluorescence emission (see *3* for details). Solid symbols correspond to data obtained in the absence of GdnHCl, and open symbols represent results obtained in the presence of 0.4 M GdnHCl. Protein concentrations were 0.64 μM (*circles*) and 6.4 μM (*squares*).

of 406 bars was observed. The corresponding value of ΔV_c, calculated according to Eq. 6, is 138 ml/mol. This value should be compared with the ΔV_p value of 96 ml/mol determined from the data obtained in the presence of GdnHCl according to Eq. 5. The γ value thus calculated equals 0.70, in the range of values exhibited by dimers that show predominantly stochastic behavior (Fig. 1).

The approach we have used here is similar to that used by Weber and co-workers (21) in the investigation of the protein concentration dependence of pressure dissociation of viruses. Viral dissociation by pressure was found to be a deterministic process, virtually independent of protein concentration. Addition of subdenaturing concentrations of urea partially restored the concentration dependence. This was interpreted as resulting from a ''homogenizing'' effect of urea on the properties of the viral particles, making the ensemble of particles more homogeneous in terms of the energetics of subunit association and converting a deterministic process into a limited stochastic equilibrium (21). In that study, however, Weber and co-workers proposed that it was the order (i.e., the number of subunits) of the oligomer that determined the behavior of the molecules under pressure dissociation, with dimers being expected to exhibit fully stochastic equilibria and large multimers such as viruses being deterministic. We now propose that, rather than simply the order of the protein oligomer, the free energy of subunit association dictates the transition from stochastic to deterministic behavior.

Biological relevance of long-lived conformational isomerism of proteins

Slow dynamics of subunit association/dissociation and interconversion between different conformational states are essential prerequisites for conformational isomerism and the so-called deterministic behavior. The examples of deterministic behavior described in this report are all observed in the transition range of pressure-induced dissociation curves. In this regard, it is important to note that the Brønsted relationship predicts that the activation free-energy barriers under physiological conditions, in the absence of pressure or any other perturbant, ought to be even higher. This may further contribute to exacerbate the anomalous behavior exhibited by those proteins.

Many biologically relevant processes such as catalysis, metabolic protein turnover, antigen presentation, and viral infection occur on timescales that are comparable to or shorter than the slow dynamics that lead to deterministic behavior. This means that, at least under certain circumstances, those biological processes may be carried out not by homogeneous protein populations, but rather by heterogeneous ensembles of oligomers that exhibit long-lived conformational isomerism.

In the case of the stability of viral particles, a clear need for a distribution of conformations that can be regarded as ''frozen'' (i.e., noninterconverting) in time has been pointed out (12,13). A typical virus shell consists of the noncovalent assembly of dozens of copies of one or a few types of coat proteins. Equation 5 predicts that the thermodynamic stability of the virus particle should be sharply dependent on protein concentration. In an infected cell, the constituent virus coat proteins are overexpressed, and it is thermodynamically favorable to assemble a virus particle. However, after cell lysis and release of the virus into the environment (with a very large dilution effect), its concentration becomes so low that each virus can be effectively considered a single, isolated particle. This means that if a viral particle were to dissociate under these conditions, the stochastic chance of subunit reassociation would be extremely small. Therefore, the rate of subunit dissociation (i.e., capsid disassembly) has to be negligibly slow (with a corresponding very high activation free energy of dissociation) to allow long-term survival of the assembled viral particle. Thus, despite the fact that virus association is thermodynamically unstable under these conditions (Eq. 5), the viral particle persists as a ''frozen'' macromolecular species, analogous to a macroscopic body, rather than as an assembly that is in dynamic chemical equilibrium with its constituent monomers.

For proteins in general, adopting a long-lived folded or oligomeric structure may be beneficial by providing increased resistance toward chemical modifications such as thiol oxidation, deamidation, or proteolytic digestion. Unfolded or unstructured conformations are in general more vulnerable to those covalent modifications (22). Indeed, the marked resistance of native TIM against thiol oxidation (3) and proteolysis (23,24) is noteworthy among the nine dimers featured in Fig. 1. Dissociation/unfolding abolishes this resistance (3). In support of this notion, it has been shown that the biological activity of TIM depends more on the association of the monomers in the dimer than on the folding of individual monomers (25).

The in vivo importance of deterministic processes is emphasized by the observation that, during evolution, functional protein assemblies have become increasingly complex (26). In fact, all nine dimers examined here have been reported to function as part of larger complexes. R17 dimer is an integral part of a virus capsid, whereas arc repressor is functional as a tetramer bound to DNA, and Ca^{2+}-ATPase is embedded in the cellular plasma membrane. Rubisco in higher order plants is found as a hexadecamer, rather than the bacterial dimer for which data are used here. The metabolic enzymes tryptophan synthase, hexokinase, enolase, and TIM have been reported to be part of sequential substrate tunneling enzyme complexes in eukaryotes (26). Thus, it could be envisaged that cellular biochemistry evolved from a prokaryotic stage dictated mainly by stochastic diffusion of both enzymes and substrates to the eukaryotic highly organized complexes comparable to macroscopic factory assembly lines. Conceptually, the constituent proteins of these complexes hold fixed positions that are not in stochastic exchange with their free forms, which demands a deterministic, macroscopic description of their stabilities.

Direct experimental support to the existence of long-lived conformers of rabbit TIM in solution has been provided by the determination of the crystallographic structure of this enzyme (27). In most TIM structures that have been solved so far, the active site loop is either observed in an open conformation in the absence of active-site ligands or in a closed conformation in the presence of ligands. The structure of rabbit TIM, however, shows that, in the absence of ligands, the active site loop is either in the open or closed conformation in different enzyme subunits (27). These structural considerations may also have interesting implications for structure-based development of drugs that perturb protein association by binding at subunit interfaces. For example, TIM from pathogenic organisms has been proposed as a potential candidate for drugs that target the subunit interface of the dimer (28,29). In this regard, it should be borne in mind that a large free energy of interaction of a drug with its target site is a necessary but not sufficient criterion for effectiveness. In addition, the dynamics of exposure of the subunit interface by transient dissociation of the oligomer should be sufficient to allow access of the drug to this site. For oligomers that exhibit the type of anomalous dissociation behavior described here, this type of approach may be hampered by the slow rates of subunit dissociation.

Finally, deterministic behavior and long-lived conformational heterogeneity of proteins may be relevant to our understanding of important human conformational diseases, including Alzheimer's and Parkinson's diseases and the prion-related transmissible spongiform encephalopathies (TSEs). It is believed that these pathological conditions are related to conformational changes from nontoxic to toxic forms of specific proteins or fragments. For example, TSEs are associated with the accumulation in the brain of an abnormal protease-resistant form of the prion protein (PrP). The disease appears to be caused by a conformational change from a benign cellular conformation of the prion protein (PrP^{C}) to a neurotoxic form (PrP^{Sc}). The molecular/energetic bases of PrP conformational change and disease transmission are still incompletely understood, and little is known of the molecular state of the protein that corresponds to the infectious, self-propagating particle. A very interesting feature of the prion hypothesis concerns the existence of prion ''strains'', i.e., clinically distinct disease forms within a single animal species that are not associated with mutations in the PrP gene. For example, eight different strains have been reported that propagate in the hamster (30). This suggests that prion molecules exhibit persistent long-lived conformational heterogeneity, which allows different strains to be perpetuated over long periods of time. Thus, the origin of prion strains may reside in the existence of multiple conformations of PrP separated by sufficiently high activation free-energy barriers (31). This is reminiscent of our recent report of the existence of long-lived ''strong'' and ''weak'' TIM dimers, which can be isolated in solution and differ markedly in their sensitivities to GdHCl dissociation (5). Consistent with this notion, a recent study has shown that, in the absence of any genetic differences, different strains of hamster prions exhibit different sensitivities to unfolding by GdnHCl (30). This is completely analogous to the behavior we have described for TIM (3–5) and indicates the deterministic nature of prions.

This work is dedicated to the memory of Prof. Gregorio Weber, whose ideas and research have inspired much of this work.

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