Protein Science (1994), *3*:2167-2174. Cambridge University Press. Printed in the USA.

REVIEW

Conformational stability of dimeric proteins: Quantitative studies by equilibrium denaturation

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(RECEIVED April 29, 1994; ACCEPTED August 22, 1994)

Abstract

The conformational stability of dimeric globular proteins can be measured by equilibrium denaturation studies in solvents such as guanidine hydrochloride or urea. Many dimeric proteins denature with a 2-state equilibrium transition, whereas others have stable intermediates in the process. For those proteins showing a single transition of native dimer to denatured monomer, the conformational stabilities, $\Delta G_u(H_2O)$, range from 10 to 27 kcal/mol, which is significantly greater than the conformational stability found for monomeric proteins. The relative contribution of quaternary interactions to the overall stability of the dimer can be estimated by comparing $\Delta G_u(H_2O)$ from equilibrium denaturation studies to the free energy associated with simple dissociation in the absence of denaturant. In many cases the large stabilization energy of dimers is primarily due to the intersubunit interactions and thus gives a rationale for the formation of oligomers. The magnitude of the conformational stability is related to the size of the polypeptide in the subunit and depends upon the type of structure in the subunit interface. The practical use, interpretation, and utility of estimation of conformational stability of dimers by equilibrium denaturation methods are discussed.

Keywords: conformational stability; dimeric proteins; equilibrium denaturation

Equilibrium denaturation studies have been very useful in understanding the structure, stabilization, and folding of small, monomeric proteins (Tanford, 1968; Pace, 1986, 1990; Timasheff, 1992). The methods for analyzing the energetics of reversible unfolding of proteins by thermal or chemical denaturant techniques (Privalov, 1979; Pace, 1986; Privalov & Gill, 1988) and the conclusions that can be drawn from thorough analysis of a few proteins (Pace, 1986, 1990; Pace et al., 1990) have been presented. Many of these studies have been done with proteins whose disulfide bonds are intact, but entropic effects of the disulfide crosslinks have also been estimated (Pace, 1990). The range of stabilities calculated for monomers is between 6 and 14 kcal/mol and represents the small difference between multiple noncovalent interactions favoring the folded protein structure and unfavorable entropic terms. Such studies have been particularly useful for analysis of packing forces in protein interiors (Alber & Matthews, 1987; Matsumura et al., 1988), the testing of the globular folding of mutant proteins (Fersht et al., 1992), and the functional interaction of residues (Carter et al., 1984). However, application of similar techniques to oligomeric proteins has, until recently, been less common, despite the potential for providing additional information on subunit interactions. Classically, the kinetics of folding pathways have been used to determine the relationship of folding to activity of oligomeric proteins and to obtain information on the folding process itself (Jaenicke, 1987; Garel, 1992; Price, 1992). On the other hand, the inherent thermodynamics of conformational stability of these oligomeric proteins can be deduced from equilibrium denaturation studies. This minireview will discuss conclusions from recent studies of dimeric proteins and the problems of interpretation, with an emphasis on those dimers showing 2-state behavior by chemical denaturation. Many studies documenting thermal denaturation simply by transition temperatures, T_m, are not covered. For a broader perspective other excellent reviews that deal with the kinetics of protein denaturation, the energetics of protein stability, temperature and pressure denaturation, and more complex oligomeric systems include Tanford (1968), Schellman (1978), Privalov (1979), Kim and Baldwin (1982), Pace (1986, 1990), Jaenicke (1987), Privalov and Gill (1988), Chothia and Finkelstein (1990), Creighton (1992), Garel (1992), Murphy and Freire (1992), Price (1992), Timasheff (1992), Barrick and Baldwin (1993), and Matthews (1993).

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Denaturation of monomers

The basis for the thermodynamic analysis of the equilibrium denaturation of monomeric proteins is the assumption that only 2 states, the native and denatured monomer, exist *at equilibrium* so that the reaction in the presence of denaturant or elevated temperatures can be described by Equation 1. For many years this equilibrium assumption, which is valid in many instances, contributed to the misunderstanding of folding pathways, which are now recognized to quite clearly have multiple *kinetic* intermediates (Kim & Baldwin, 1982; Barrick & Baldwin, 1993).

$$N \rightleftharpoons D. \quad (1)$$

If the fraction of denatured species can be measured by suitable biophysical or spectroscopic techniques (e.g., fluorescence, CD, NMR, absorption), then the free energy of the reaction at any denaturant concentration or any temperature can be calculated from the equilibrium constant, $K_u = f_d/f_n$; where f_d and f_n are the fraction of denatured and native protein, respectively. Extrapolation (usually linear) of $\ln(K_u)$ to zero denaturant concentration provides the energy of stabilization in H_2O (actually dilute buffer), $\Delta G_u(H_2O)$ (Schellman, 1978; Pace, 1986; Pace et al., 1990; Santoro & Bolen, 1992). Although the extrapolation can be done graphically (Pace, 1986), a number of computer programs have been developed to fit the raw data (Gittelman & Matthews, 1990; Pace et al., 1990; Liang & Terwilliger, 1991; Timm & Neet, 1992; Clark et al., 1993; Mann et al., 1993; Morjana et al., 1993). The effects of denaturants thus reveal energetics that exist in the protein and can be calculated for the normal situation in the absence of denaturant. Thermal denaturation experiments provide the transition temperature, T_m, and the enthalpy of unfolding, ΔH_u, at the transition temperature (Privalov & Gill, 1988). Knowledge of the heat capacity change for the reaction, ΔC_p, which is usually obtained from calorimetric experiments (Privalov & Potekhin, 1986) or from combined thermal and chemical denaturation data, then allows calculation of ΔH_u and ΔG_u for the denaturation of the protein at any temperature (e.g., Bowie & Sauer, 1989a; De Francesco et al., 1991; Steif et al., 1993). In other cases, quantitative thermodynamic measurements have been made on proteins that unfold upon lowering the temperature (e.g., Privalov et al., 1986; Chen & Schellman, 1989).

Three-state dimer denaturation

With dimeric (or oligomeric) proteins additional modes of stabilization are available at the quaternary structural level. The contribution of intrachain and interchain interactions to the overall protein conformational stability can make significantly distinct contributions. A general 3-state model of the equilibrium dissociation and unfolding of the dimeric protein involving native dimer (N_2), native monomer (N) or a monomeric intermediate (I), and denatured monomer (D) is described by Equation 2. Completely unfolded dimers (D_2) are not likely to exist (except in cases of nonspecific aggregation of unfolded intermediates or covalently linked dimers), but evidence for partially unfolded dimeric intermediates (I_2) is occasionally reported (e.g., Blackburn & Noltmann, 1981). Provision is, however, made for a native-like or compact monomeric intermediate, I, for reasons discussed below. Of course, conditions could occur in which multiple stable intermediates might give rise to more complex, multistate denaturation (Herold & Kirschner, 1990).

$$N_2 \overset{K_1}{\rightleftharpoons} 2N \overset{K_2}{\rightleftharpoons} 2D \text{ (a)} \quad \text{or} \quad N_2 \overset{K_1}{\rightleftharpoons} 2I \overset{K_2}{\rightleftharpoons} 2D \text{ (b)} \quad (2)$$

$$K_1 = [I]^2/[N_2]; \; K_2 = [D]/[I]. \quad (3)$$

Two distinct equilibrium constants describe each reaction (Equation 3); for convenience, only I is used to represent the monomeric intermediate. The dimeric dissociation constant, K_{dis} ($=[N]^2/[N_2]$) obtained by dilution is often assumed to be equivalent to K_1 but in reality can represent more complex equilibria depending on the magnitude of K_2 (see below). The overall equilibrium constant for denaturation, K_u, is equal to $K_1K_2^2$ and the overall free energy of unfolding, ΔG_u, can be partitioned into analogous energetic terms, $\Delta G_u = \Delta G_1 + 2\Delta G_2$ (from Equation 2). At one extreme, for proteins with K_1 significantly larger than K_2, the quaternary interactions stabilizing dimeric association will be less than the secondary and tertiary interactions stabilizing the folded monomer. Conditions will, therefore, exist in which an intermediate is populated to an extent comparable to that of the native dimer or denatured monomer. The compact monomeric intermediate structure may not be identical to the conformation of the subunits in the native dimer, but some native-like secondary or tertiary folding may exist. A concentration dependence of responses that measure the unfolding reaction will normally be observed due to the coupling between K_1 and K_2.

Numerous examples exist of compact, monomeric intermediates that are significantly populated during the equilibrium denaturation of a dimer. Some examples include the β_2 subunit of tryptophan synthetase (Zetina & Goldberg, 1980), λ repressor (Pabo et al., 1979; Banik et al., 1992), aspartate amino transferase (Herold & Kirschner, 1990; Leistler et al., 1992), phosphoglucose isomerase (Blackburn & Noltmann, 1981), superoxide dismutase (Mei et al., 1992), and glutathione *S*-transferase (Aceto et al., 1992; Sacchetta et al., 1993). In general, the intermediates have been detected indirectly by noncoincidental denaturation curves measured by methods that are sensitive to distinct structural features. Thus, the Trp environment detected by fluorescence may change at a different urea concentration than the secondary structure revealed by CD or the quaternary structure revealed by gel filtration. A frequent theme in these studies is that the protein is larger and has domains that unfold independently of each other and of the quaternary interactions (Zetina & Goldberg, 1980; Banik et al., 1992), thus giving rise to a structure of intermediate stability. In other cases, the structures of the monomeric intermediates do not have exactly the same spectral properties as the native subunits or domains of the dimer and may, therefore, represent a collapsed form or molten globule intermediate (Herold & Kirschner, 1990; Mei et al., 1992). Intrachain interactions are sufficient in these cases to stabilize a compact structure with α-helices and β-sheets in the absence of interchain interactions. In some cases (Blackburn & Noltmann, 1981; Aceto et al., 1992; Clark et al., 1993; Sacchetta et al., 1993), a partially unfolded dimer has also been proposed as an intermediate. Because molten globules are frequently found in denaturation and refolding of monomers (Barrick & Baldwin, 1993), it should not be surprising that compact intermediates with secondary structure also occur as discrete forms in equilibrium denaturation of dimeric proteins.

Two-state dimer denaturation

Although dimeric proteins have additional modes of quaternary structure stabilization, many still follow a 2-state transition, representing the other extreme of Equation 2 in which K_2 is significantly larger than K_1. Dissociation, therefore, leads to the formation of an intrinsically unstable species and a folded monomeric species will not be significantly populated at equilibrium; in this extremity the model reduces to a dimeric, 2-state model (Equation 4) and the energetics can be readily determined in a manner similar to that for monomer denaturation.

$$N_2 \rightleftharpoons 2D \tag{4}$$

$$K_U = [D]^2/[N_2] = 2P_t[f_d^2/(1 - f_d)]. \tag{5}$$

The 2 states are now a native dimer, N_2, and a denatured monomer, D. The fraction of denatured protein can be measured spectroscopically as with monomers. However, the equilibrium constant must now be calculated with Equation 5, which shows the dependence of K_u on the fraction of denatured protein, f_d, and on the total protein concentration, P_t (normally in monomer units). Evaluation of K_u and extrapolation of $\ln(K_u)$ to standard conditions (absence of denaturant) to obtain $\Delta G_u(H_2O)$ can then be performed as described for monomeric proteins. The 2-state model implies that native monomer (or a monomeric intermediate) does not exist at significant concentrations at equilibrium, i.e., that quaternary interactions are necessary for stabilization of the folded monomeric state. However, a partially structured monomer or denatured dimer could still occur transiently and be observed by kinetic measurements. Considerations of the intramolecular interactions in the crystal structure of the dimers of gene V protein (Brayer & McPherson, 1983), nerve growth factor (NGF; McDonald et al., 1991), Arc repressor (Raumann et al., 1994), and Trp repressor (Zhang et al., 1987) have led to independent suggestions that the isolated monomer would not be able to retain its folded conformation. Evidence from pressure denaturation studies has also been presented for an equilibrium between the Arc repressor dimer and a molten globule monomer, in which intersubunit β-sheets have been replaced by intramolecular β-strand interactions (Silva et al., 1992; Peng et al., 1993). The report by Bowie and Sauer (1989a) on dimeric Arc repressor initiated a series of similar analyses on other dimeric proteins. The conformational stabilities from these studies of dimeric proteins undergoing a 2-state equilibrium denaturation are summarized in Table 1, along with simple dissociation constants and enthalpies of stability, where available.

Experimental considerations and basic interpretations

In order to determine the true 2-state nature of the denaturation transition, different biophysical methods should be used that are sensitive to distinct structural features. Tests for the 2-state nature of the dimer dissociation are critical and should be made extensively with different protein concentrations and conditions. Tryptophan fluorescence (static, dynamic, and/or anisotropic) to detect tertiary structural changes is frequently used in conjunction with CD to assess secondary structure (Bowie & Sauer, 1989a; Reece et al., 1991; Timm & Neet, 1992; Mann et al., 1993). NMR is powerful but less often used (Raleigh & DeGrado, 1992). Activity measurements are an indirect assessment of structure that are occasionally utilized (Dirr & Reinemer, 1991). The dependence of these techniques on protein concentration is a unique characteristic of the coupled denaturation and dissociation of *oligomeric* protein systems (2- or 3-state) and should be used as a definitive criterion for assigning the type of system as well as for evaluating the thermodynamic parameters (Bowie & Sauer, 1989a; Gittelman & Matthews, 1990; De Francesco et al., 1991; Liang & Terwilliger, 1991; Reece et al., 1991; Grant et al., 1992; Monera et al., 1992; Timm & Neet, 1992; Thompson et al., 1993; Timm et al., 1994). Alternatively, methods that directly determine the association state, such as ultracentrifugation or gel filtration, can directly detect the dissociation step. For example, with cyclic AMP receptor protein (CRP) 3 spectroscopic techniques were coincident and nearly independent of CRP concentration, thus revealing that the dissociation detected by sedimentation equilibrium was not coupled to denaturation (Cheng et al., 1993).

The extrapolation of ΔG to zero denaturant concentration for the 2-state dimer (Equations 4, 5) is more complex than the equivalent extrapolation for the 2-state monomer (Equation 1). The validity of the monomeric extrapolation has been well tested and discussed (Schellman, 1978; Pace, 1986; Pace et al., 1990; Santoro & Bolen, 1992; Timasheff, 1992); however, with the dimer the extrapolation tacitly assumes that effects of denaturant on both unfolding and dissociation reactions are linear. In several cases, the effects of urea and guanidine have provided $\Delta G_u(H_2O)$ values very close together (Bowie & Sauer, 1989a; Dirr & Reinemer, 1991; Timm & Neet, 1992), thus providing confidence that the linear assumptions for these dimers are valid. Furthermore, for thermodynamic measurements, it is essential that the system be fully reversible and that no hysteresis occur in comparison of denaturation and renaturation curves.

The midpoint of a thermal (Pakula & Sauer, 1989) or chemical (Bowie & Sauer, 1989b) denaturation transition can also be used to compare a series of related proteins. The parameter, *Cm* or Gdn50, is the denaturant concentration when $f_d = f_n$, but is a function of protein concentration for dimer coupled systems (Bowie & Sauer, 1989a; Timm & Neet, 1992). The transition midpoint for chemical denaturation is related to the other parameters by $\text{Gdn50} = \{RT\ln[\text{protein}] + \Delta G_u(H_2O)\}/m$, where m is the slope of the ΔG versus [Gdn] plot. The *difference* in the midpoint between 2 curves for different mutants or conditions, ΔGdn50, is related to the difference in free energy, but the value of m cannot be assumed to be constant under different conditions. In general, it is more informative to work with true extrapolated $\Delta G_u(H_2O)$ values, even if there is slightly more error than in the determination of Gdn50.

An additional concern in the study of both dimeric and monomeric denaturation is the nature of the unfolded state. Any residual protein structure remaining at high concentrations of the denaturant could provide an artificially low $\Delta G_u(H_2O)$ value and still contribute to stability of the native state. This problem is not well addressed, in general, although in certain instances residual structure in the solvent has been measured by NMR (Peng et al., 1993). Most of the dimers in Table 1 lack disulfide bonds with the exception of the neurotrophins (NGF, brain-derived neurotrophic factor [BDNF], neurotrophin [NT]-3, NT-4/5) and therefore might be expected to have less residual structure in the denatured state. However, most monomers that have been extensively studied, such as ribonuclease and lyso-

Table 1. *Conformational stabilities of dimeric proteins fitting a two-state denaturation*

	$\Delta G_u(H_2O)$ (kcal/mol)[a]	ΔG_{dis} (kcal/mol)	ΔH_u (kcal/mol)	C_p (kcal/mol °C)	Size (no. residues)	Reference
Growth hormone (human pituitary)	27.8[b]	4.7			191	Cunningham et al. (1991)
Brain-derived neurotrophic factor (BDNF) (human)	26.4 ± 2.4				119	Timm et al. (1994)
Glutathione *S*-transferase (porcine lung)	25.3 ± 2				207	Dirr and Reinemer (1991)
Neurotrophin-3 (NT-3) (human)	22.7 ± 1.3				119	Timm et al. (1994)
Neurotrophin-4/5 (NT-4/5) (human)	20.8 ± 1.2				130	Timm et al. (1994)
Nerve growth factor (NGF) (mouse)	19.3 ± 1	>16			118	Timm and Neet (1992)
cAMP receptor protein (CRP) (*E. coli*)	19.2 ± 0.6[c]	12.0 ± 0.6			209	Cheng et al. (1993)
Trp aporepressor (*E. coli*)	18.2 ± 2				107	Mann et al. (1993); Fernando and Royer (1992)
Repressor of primer (ROP) (ColE1 plasmid, *E. coli*)	17.1		138.6 @ 71 °C	2.5	63	Steif et al. (1993)
Gene V protein (bacteriophage f1)	16.3 ± 0.7				87	Liang and Terwilliger (1991)
HIV-1 protease (human immunodeficiency virus-1)	14 ± 1	12[d]			99	Grant et al. (1992)
Dihydrofolate reductase-II (R67) (R-plasmid)	13.9 ± 1.1				78	Reece et al. (1991)
SIV protease (simian immunodeficiency virus)	13 ± 1				99	Grant et al. (1992)
α_2(PRR) (artificial)	12.8				35	Ho and DeGrado (1987)
LFB1 transcription factor dimerization domain (rat liver)	11.5[e]		50 @ 65 °C	0.74	32	De Francesco et al. (1991)
Troponin C Ca^{2+} binding III peptide (LFIL) (skeletal muscle)	11		7.8		34	Monera et al. (1992)
Arc repressor (bacteriophage p22)	9.5 ± 0.3	9.7–10.2	71 @ 54 °C	1.6	53	Bowie and Sauer (1989a); Milla and Sauer (1994); Silva et al. (1992)

[a] All values obtained between pH 6 and 7.6 and 25 or 30 °C, except for DHFR R67 at pH 5.
[b] $\Delta G_u(H_2O)$ calculated from data in the reference.
[c] A 3-state transition with the overall $\Delta G_u(H_2O)$ calculated by addition of ΔG_1 (12 kcal/mol) and ΔG_2 (7.2 kcal/mol) determined independently (Cheng et al., 1993).
[d] ΔG_{dis} extrapolated to pH 6, 25 °C from Darke et al. (1994).
[e] ΔG_{dis} = 14–16 kcal/mol for intact LFB1 (Nicosia et al., 1990).

zyme, do have disulfides (Pace, 1990). For comparison of mutants or of solvent conditions, the effect of possible structural remnants in the denaturing condition should be considered as well as differences in conformational stability of the native state.

Energetic aspects of dimer denaturation

Comparison of $\Delta G_u(H_2O)$ to the simple dissociation of native dimers, ΔG_{dis}, independently determined by dissociation, can provide information on the relative contributions of tertiary and quaternary structure to stability. In the few instances where adequate data are available (Table 1), estimation of the stability of the isolated monomer can be made. The putative "native" monomer of NGF, human immunodeficiency virus (HIV)-1 protease, and the troponin C peptide are calculated to be stabilized by only 1–2 kcal/mol monomer from this difference in ΔG values (Table 1). The $\Delta G_u(H_2O)$ and ΔG_{dis} for Arc repressor are essentially equivalent, suggesting that the isolated monomer is not stabilized at all. These values for monomer stabilization are well below the normal conformational stability of true monomers of 6–14 kcal/mol (Pace, 1990) and support the concept that a major contribution of the dimer is to maintain the "native" or active conformation of the subunits in these proteins. In contrast, human growth hormone, which binds to receptor as a monomer, only weakly dimerizes in the absence of Zn^{2+} with a dissociation constant of 0.4 mM (Cunningham et al., 1991); growth hormone has an overall high thermodynamic stability, 27.8 kcal/mol (Table 1), but a large proportion (83%) comes from the stabilization of the pair of monomers, 11.5 kcal/mol for each of 2 monomers (Cunningham et al., 1991). Dimeric β-lactoglobulin, which was shown in classic denaturation studies to dissociate at lower urea concentrations than required for unfolding (Pace & Tanford, 1968), would be another example of this latter situation.

Interesting evolutionary questions are raised by these energy considerations of dimeric proteins. Based on the dimers that have a significant proportion of the overall stability contributed by the quaternary contribution, i.e., NGF, HIV-1 protease, and Arc repressor, one could argue that the quaternary structure has evolved for the benefit of the overall conformation of these proteins, i.e., the "native" conformation of the monomer could not have evolved without the stabilization provided by the dimeric

configuration. On the other hand, the HIV-1 protease requires both subunits to form the catalytic site and the Arc repressor requires both subunits to bind to a symmetrical dyad DNA binding site; stability and function are strongly correlated in these 2 proteins. Whether the monomer evolved a putative activity and then acquired quaternary stabilization or whether a stable dimer formed early and subsequently evolved function are evolutionary questions remaining to be answered.

From these comparisons of energy partitioning, one might conclude that quaternary interactions can provide 25–100% of the conformational stability in proteins that can dimerize. Strictly speaking, this calculation for dimers is only true at a standard state concentration of 1 M monomer and dimer, i.e., ΔG_0. Nevertheless, the thermodynamic energy defines the quantitative limits of the potential molecular interaction and allows consideration of contributing features to overall stability of oligomeric proteins. At concentrations of 1 pM to 1 μM, where proteins usually function and are studied, substantial dissociation may occur leading to a diminished *effective* contribution of the quaternary interaction energy.

Consideration of these concentration effects leads to the surprising conclusion that the meaning of ΔG_{dis} and its expected relationship to $\Delta G_u(H_2O)$ depends on the stability of the monomer in the system. Normally, denaturation/dissociation should lead to a denatured monomer ($\Delta G_u = \Delta G_1 + 2\Delta G_2$). On the other hand, one would expect that dissociation by dilution would lead to a monomer in its native conformation ($\Delta G_{dis} = \Delta G_1$), but this would be explicitly true only when $\Delta G_2 \ll \Delta G_1$. In those cases where the stability of the monomer is relatively low, e.g., Arc, NGF, HIV-1 protease, and troponin C peptide (Table 1), the ratio of the native monomer to denatured monomer, dictated by the magnitude of ΔG_2, is low. Thus, ΔG_{dis} actually measures the dissociation coupled to unfolding, i.e., ΔG_{dis} is equal to $\Delta G_1 + 2\Delta G_2$, and approaches ΔG_u as ΔG_2 becomes significant in magnitude relative to ΔG_1 (where all of the ΔG values are extrapolated to aqueous solution). This situation holds for the Arc repressor in which $\Delta G_u = \Delta G_{dis}$.

Simulation studies of Equation 5 at fixed ΔG and P_t values were used to further explore the relationship between putative equilibrium states. For dimeric, 2-state proteins at the low end of stability in Table 1, i.e., 10–12 kcal/mol, less than 2% of the protein will exist as a folded dimer in the concentration range below 1 nM. As correctly deduced for Arc repressor (Bowie & Sauer, 1989a), at the concentration of half saturation for DNA binding, 500 pM, the protein would be mainly an unfolded monomer even though it binds to the DNA as a folded dimer. For proteins in the middle range of stability, e.g., 15 kcal/mol, 40% native dimer will exist at 10 pM and 70% at 100 pM. Only for those proteins with inherent stabilities of about 20 kcal/mol or above will the protein be fully native dimer at physiological concentrations below 10 pM. Alternatively, if ΔG_2 is small so that the "native" monomer is relatively stable, then dissociation may occur at low physiological concentrations, but native-like monomer would predominate. This situation may occur with some of the proteins that undergo a 3-state denaturation transition (discussed above).

For the 2-state system, K_u, the free energy of stabilization, can be readily measured as described, but measurement of both K_1 and K_2 for the 3-state systems is more difficult. Three different procedures can be used. (1) A biphasic spectroscopic curve found for luciferase (Clark et al., 1993) has been directly fit to a 3-state model to yield values for both K_1 and K_2. (2) Alternatively, sets of noncoincident spectroscopic data (CD, fluorescence intensity, and UV absorption at 292 nm) for point mutants of the Trp aporepressor have been analyzed by simultaneous global fitting to obtain both K_1 and K_2 (Mann et al., 1993). (3) The K_{dis} of CRP has been measured by sedimentation equilibrium in guanidine solutions and extrapolated to zero denaturant concentration in concert with spectroscopic measurements. This measurement then allowed determination of K_2 from the spectroscopic data. Despite the coincidence of steady-state fluorescence intensity, fluorescence anisotropy, and CD, the molecular weight measurements quite clearly showed that dissociation occurred before the conformational change (Cheng et al., 1993). The overall $\Delta G_u(H_2O)$ for CRP is shown in Table 1, with the individual K_1 and K_2 parameters reported in the footnote. Comparison of K_1 and K_2 from these determinations can directly assess the contribution of tertiary and quaternary structure to stability without the need for a separate estimate of K_{dis}. As befitting a 3-state dimer system, the monomer of CRP is stabilized by 7.2 kcal/mol and contributes about 35% of the overall stability of the dimer.

Structural aspects of dimer denaturation

The data in Table 1 suggest that a relationship may exist between the size of the dimer and the tertiary/quaternary stabilization. Indeed, a roughly linear correlation exists between the number of amino acid residues (N) in the monomer and the value of $\Delta G_u(H_2O)$ (an equation of $\Delta G_u(H_2O) = 8.8 + 0.08N$ with a correlation coefficient of 0.6). Obvious exceptions exist such as NGF and BDNF, which are essentially the same size, but differ by a significant 7 kcal/mol in stability (Timm et al., 1994). The size relationship is, perhaps, not surprising because the potential surface area for interaction between subunits in a dimer increases with mass. Chothia and coworkers have shown that the solvent-accessible surface area is correlated with molecular mass of oligomers (Miller et al., 1987) and suggested, on theoretical grounds (Chothia et al., 1976; Janin et al., 1988), that the association energy is related to the surface area of the buried interface of an oligomer, usually taken as 25 cal/Å^2 for hydrophobic interactions. Moreover, the thermal stability of *monomers* tends to be proportional to protein size, because ΔH or C_p *per residue* is approximately constant (within 15%) at 1.5 cal·(mol amino acid)$^{-1}$ and 14 cal·(°C·mol amino acid)$^{-1}$, respectively (Privalov & Gill, 1988). We have demonstrated here (Table 1) an approximate correlation between molecular mass and the experimentally determined thermodynamic stability of an oligomeric series. A more exact calculation would take into account the area and composition of the subunit interfaces for these proteins. This correlation for dimers is over a rather narrow range of protein sizes and may not be valid with larger oligomeric proteins.

The structure of the interface between the dimeric subunits of many of the proteins of Table 1 has been determined from crystallographic studies, providing relevant information on the conformational stabilization of these dimers. Amphiphilic α-helices in a coiled-coil or leucine-zipper comprise the intersubunit interaction in LFB1, α_2(PRR), repressor of primer (ROP), and the primary interaction in CRP. HIV-1 protease, simian immunodeficiency virus (SIV) protease, and the R67 dihydrofolate reductase (DHFR) have primarily β-sheets stabilizing

interactions across the subunit interface. The gene V protein and the neurotrophins (NGF, BDNF, NT-3, NT-4/5) have hydrophobic side chains from β-strands that stabilize the interface. Three other proteins have combinations of secondary structures at the interface that are highly entwined α-helices (Trp aporepressor), combined α, β, and hydrophobic interactions (Arc repressor), and an interacting helix-loop-helix motif (troponin C LFIL). From these results one concludes that stabilization over a broad range of energies can be achieved by various types of dimer interfaces.

Effects of environment on stability

As would be expected, the stability of a protein can be increased by ligand binding. Addition of a peptide inhibitor shifts the stability of the HIV-1 protease by about 5.3 kcal/mol (Grant et al., 1992) and the addition of tryptophan to the Trp repressor shifts the measured stability by 6 kcal/mol (Fernando & Royer, 1992). Zn^{2+} shifts the overall conformational stability, ΔG_u, and the pure dissociation reaction, ΔG_{dis}, of growth hormone by 1.5–3 kcal/mol (Cunningham et al., 1991).

For the proteins of Table 1 that have been tested, destabilization toward denaturation occurs at lower pH values. This effect can be as large as 5 kcal/mol. The studies of $\Delta G_u(H_2O)$ as a function of pH for dimeric NGF (Timm et al., 1994) show a remarkable similarity to the pH dependency of (monomeric) RNase A (Pace et al., 1990). The effect of folding on the pK of a limited number of ionizable groups—5 in RNase A and 6 or less in NGF—appear to account for most of the pH dependency (Pace et al., 1990; Timm et al., 1994) of $\Delta G_u(H_2O)$. Moreover, the value of m (the dependence of ΔG_u on denaturant concentration) decreased with decreasing pH for both RNase A and NGF in guanidine (Pace et al., 1990; Timm et al., 1994). In the former case, the pH effect on m was attributed to differential accessibility of side chains in the unfolded state, whereas in the latter case deviations from the 2-state ($N_2 = 2D$) model with NGF at low pH were proposed. These results confirm the notion that charge–charge interaction can be an important factor in stabilizing dimeric proteins.

Effects of structural alterations on stability

Several investigations have compared the stability of the proteins of Table 1 after mutation or modification. Two-residue substitutions in either the N-terminal or the C-terminal region of a 34-residue peptide from troponin C decrease the conformational stability of the dimeric peptide by 4 kcal/mol (Monera et al., 1992). Deletion of 16 residues from the N-terminus of the 78-residue R67 DHFR peptide decreases the stability by 2.6 kcal/mol (Reece et al., 1991). Human and mouse NGF differ in 12 of 118 residues but differ by 4 kcal/mol (Timm et al., 1994); key residues have been suggested to account for this difference. On the other hand, the HIV-1 and SIV proteases (50% identity) only differ in stability by 1 kcal/mol, despite being much more dissimilar than the NGF pair. When Arc repressor mutants with 1–5 residues substituted were selected for partial function, changes of ±0.4 to ±3 kcal/mol were found, compared to wild type (Bowie & Sauer, 1989b; see also Silva et al., 1992). Similar changes in the ΔG of stability (up to 5 kcal/mol) have been observed with extensive multiple mutations of core residues of f1 gene V protein (Sandberg & Terwilliger, 1993) and from comparisons of thermal denaturation (ΔT_m) of suppressor mutations of a λ Cro mutant protein (Pakula & Sauer, 1989). Thus, the change in stability of a dimeric protein after amino acid substitutions can be positive or negative and as large as 5 kcal/mol or less than 1 kcal/mol with the possibility of compensating effects when multiple substitutions are made. These results on dimers are not particularly different from comparisons of stabilities of mutants of monomeric proteins (Alber & Matthews, 1985; Matthews, 1987; Fersht et al., 1992; Lim et al., 1992), except that certain residues can directly affect subunit interfaces in the dimers.

Engineering of dimeric proteins, involving more extensive changes than simple amino acid substitution or truncation of the chain, has provided different information on stability. An interesting "subunit-fusion" of the gene V protein connected the N- and C-termini with a short (5–6-residue) loop to make a single polypeptide chain retaining the remainder of the dimeric structure (Liang et al., 1993). As expected from entropy considerations, the folded "monomer" was about 5 kcal/mol more stable than the wild-type dimer, primarily due to an increased rate of refolding. A stable, monomeric form of the λ Cro DNA binding protein that was unable to associate into a dimer has been engineered by rather extensive rearrangement of the subunit interface to prevent productive interactions (Mossing & Sauer, 1990). A similar scheme produced an active, monomeric form of triosephosphate isomerase (Borchert et al., 1994). Surprisingly, both monomeric Cro and the isomerase had apparently higher stabilities, based on an increased T_m of denaturation by 13° and 3°, respectively (compared to the corresponding wild-type dimer). However, quantitation using concentration-dependent equilibrium constants was not applied in either case. Clearly, information about the structure and the stability of a dimer can be utilized to engineer novel protein forms with desirable characteristics.

A fine balance may exist between a 2-state denaturation system and a 3-state denaturation with an intermediate. The DNA binding domain of CRP, produced by limited proteolytic removal of the C-terminus, has an overall stability only 0.4–1 kcal/mol less than the native protein, but the stability of the monomer fragment (as compared to the dimer interface) contributes about 1.5 kcal/mol more than in the native CRP (Cheng et al., 1993). Although the equilibrium denaturation of NGF at neutral pH occurs as a 2-state system (Timm & Neet, 1992), a compact monomeric intermediate has been proposed (Timm et al., 1994) in the low pH guanidine denaturation of NGF. This intermediate may be similar to the molten globule *kinetic* folding intermediate suggested for the related neurotrophin, BDNF (Narhi et al., 1993). Mutation of single amino acid residues of the Trp aporepressor, W19F or W99F, at the subunit interface (Zhang et al., 1987) also resulted in destabilization of the dimer relative to the monomeric intermediate and conversion of the equilibrium denaturation from a 2-state to a 3-state system with no significant effect on the overall stability (Mann et al., 1993). Noncoincidence of the CD and fluorescence data suggested that the mutation disrupted the subunit interface containing each Trp residue more than the secondary structure of the subunit. The mutant monomeric intermediate was stabilized by 8–11 kcal/mol relative to the unfolded monomer and ΔG_{dis} was sufficiently small that dissociation by dilution in the micromolar concentration range should be observable with the tryptophan mutants (Mann et al., 1993). These modifications imply that secondary

and tertiary structural interactions can contribute comparable stabilization energy as quaternary interactions, but the outcome may be quite sensitive to small changes in amino acid side-chain interactions.

Summary

Careful measurement of the thermodynamics of equilibrium denaturation of dimeric proteins can reveal characteristics of the energy stabilizing the folded protein structure. Care must be taken to establish that the protein is 2-state or multiple-state, by judicious choice of biophysical methods that reveal coincidence, or the lack thereof, of different structural transitions in the protein upon denaturation. Measurement of dissociation or determination of the concentration dependence of the spectroscopic data is necessary to confirm the coupling of the denaturation and dissociation processes for either 2- or 3-state denaturations. Utilization of both urea and guanidine (or temperature) is helpful in establishing the lack of a stabilization of an unexpected intermediate by either denaturant (Morjana et al., 1993) and in making a successful extrapolation to aqueous conditions. Caution is necessary in interpreting the nature of this extrapolation. Examination of data on proteins in the literature reveal that many, 16, appear to follow a 2-state transition and, of these, the conformational stabilization energy is dependent on both the size of the protein and the nature of the intersubunit interactions. Independent measurements of simple dissociation constants indicate that quaternary interactions can provide 65–100% of the conformational stability of protein dimers.

References

Aceto A, Caccuri AM, Sacchetta P, Bucciarelli T, Dragani B, Rosato N, Federici G, Di Ilio C. 1992. Dissociation and unfolding of Pi-class glutathione transferase. Evidence for a monomeric inactive intermediate. *Biochem J 285*:241–245.

Alber T, Matthews BW. 1987. Structure and thermal stability of phage T4 lysozyme. *Methods Enzymol 154*:511–534.

Banik U, Saha R, Mandal NC, Bhattacharyya B, Roy S. 1992. Multiphasic denaturation of the lambda repressor by urea and its implications for the repressor structure. *Eur J Biochem 206*:15–21.

Barrick D, Baldwin RL. 1993. The molten globule intermediate of apomyoglobin and the process of protein folding. *Protein Sci 2*:869–876.

Blackburn MN, Noltmann EN. 1981. Evidence for an intermediate in the denaturation and assembly of phosphoglucose isomerase. *Arch Biochem Biophys 212*:162–169.

Borchert TV, Abagyan R, Jaenicke R, Wierenga RK. 1994. Design, creation, and characterization of a stable, monomeric triosephosphate isomerase. *Proc Natl Acad Sci USA 91*:1515–1518.

Bowie JU, Sauer RT. 1989a. Equilibrium dissociation and unfolding of the Arc repressor dimer. *Biochemistry 28*:7139–7143.

Bowie JU, Sauer RT. 1989b. Identifying determinants of folding and activity for a protein of unknown structure. *Proc Natl Acad Sci USA 86*:2152–2156.

Brayer GD, McPherson A. 1983. Refined structure of the gene 5 DNA binding protein from bacteriophage fd. *J Mol Biol 169*:565–596.

Carter PJ, Winter G, Wilkinson AJ, Fersht AR. 1984. The use of double mutants to detect structural changes in the active site of the Tyr-tRNA synthetase. *Cell 38*:835–840.

Chen BL, Schellman JA. 1989. Low-temperature unfolding of a mutant of phage T4 lysozyme. 1. Equilibrium studies. *Biochemistry 28*:685–691.

Cheng X, Gonzalez ML, Lee JC. 1993. Energetics of intersubunit and intrasubunit interactions of *Escherichia coli* adenosine cyclic 3′,5′-phosphate receptor protein. *Biochemistry 32*:8130–8139.

Chothia C, Finkelstein AV. 1990. The classification and origins of protein folding patterns. *Annu Rev Biochem 59*:1007–1039.

Chothia C, Wodak S, Janin J. 1976. Role of subunit interfaces in the allosteric mechanism of hemoglobin. *Proc Natl Acad Sci USA 73*:3793–3797.

Clark AC, Sinclair JF, Baldwin TO. 1993. Folding of bacterial luciferase involves a non-native heterodimeric intermediate in equilibrium with the native enzyme and the unfolded subunits. *J Biol Chem 268*:10773–10779.

Creighton TE. 1992. *Protein folding*. New York: Freeman.

Cunningham BC, Mulkerrin MG, Wells JA. 1991. Dimerization of human growth hormone by zinc. *Science 253*:545–548.

Darke PL, Jordan SP, Hall DL, Zugay JA, Shafer JA, Kuo LC. 1994. Dissociation of the HIV-1 protease dimer subunits. Equilibrium and rates. *Biochemistry 33*:98–105.

De Francesco R, Pastore A, Vecchio G, Cortese R. 1991. Circular dichroism study on the conformational stability of the dimerization of transcription factor LFB1. *Biochemistry 30*:143–147.

Dirr HW, Reinemer P. 1991. Equilibrium unfolding of class pi glutathione *S*-transferase. *Biochem Biophys Res Commun 180*:294–300.

Fernando T, Royer CA. 1992. Unfolding of Trp repressor studied using fluorescence spectroscopic techniques. *Biochemistry 31*:6683–6691.

Fersht AR, Matouschek A, Serrano L. 1992. The folding of an enzyme. I. Theory of protein engineering analysis of stability and pathway of protein folding. *J Mol Biol 224*:771–782.

Garel JR. 1992. Folding of large proteins: Multidomain and multisubunit proteins. In: Creighton TE, ed. *Protein folding*. New York: Freeman. pp 405–454.

Gittelman MS, Matthews CR. 1990. Folding and stability of *trp* aporepressor from *E. coli*. *Biochemistry 29*:7011–7020.

Grant SK, Deckman IC, Culp JS, Minnich MD, Brooks IS, Hensley P, Debouck C, Meek TD. 1992. Use of protein unfolding studies to determine the conformational and dimeric stabilities of HIV-1 and SIV proteases. *Biochemistry 31*:9491–9501.

Herold M, Kirschner K. 1990. Reversible dissociation and unfolding of aspartate aminotransferase from *E. coli*. Characterization of a monomeric intermediate. *Biochemistry 29*:1907–1913.

Ho SP, DeGrado WF. 1987. Design of a 4-helix bundle protein. Synthesis of peptides which self-associate into a helical protein. *J Am Chem Soc 109*:6751–6758.

Jaenicke R. 1987. Folding and association of proteins. *Prog Biophys Mol Biol 49*:117–237.

Janin J, Miller S, Chothia C. 1988. Surface, subunit interfaces, and interior of oligomeric proteins. *J Mol Biol 204*:155–164.

Kim PS, Baldwin RL. 1982. Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Annu Rev Biochem 51*:459–489.

Leistler B, Herold M, Kirschner K. 1992. Collapsed intermediates in the reconstitution of dimeric aspartate aminotransferase from *Escherichia coli*. *Eur J Biochem 205*:603–611.

Liang H, Sandberg WS, Terwilliger TC. 1993. Genetic fusion of subunits of a dimeric protein substantially enhances its stability and rate of folding. *Proc Natl Acad Sci USA 90*:7010–7014.

Liang H, Terwilliger TC. 1991. Reversible denaturation of the gene V protein of bacteriophage f1. *Biochemistry 30*:2772–2782.

Lim WA, Farruggio DC, Sauer RT. 1992. Structural and energetic consequences of disruptive mutations in a protein core. *Biochemistry 31*:4324–4333.

Mann CJ, Royer CA, Matthews CR. 1993. Tryptophan replacements in the *trp* aporepressor from *E. coli*. Probing the equilibrium and kinetic folding models. *Protein Sci 2*:1853–1861.

Matsumura M, Becktel WJ, Matthews BW. 1988. Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile3. *Nature 334*:406–410.

Matthews C. 1987. Effect of point mutations on the folding of globular proteins. *Methods Enzymol 154*:498–510.

Matthews CR. 1993. Pathways of protein folding. *Annu Rev Biochem 62*: 653–683.

McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL. 1991. New protein fold revealed by a 2.3-Å resolution crystal structure of nerve growth factor. *Nature 354*:411–414.

Mei G, Rosato N, Silva N Jr, Rusch R, Gratton E, Savini I, Finazzi-Agro A. 1992. Denaturation of human Cu/Zn superoxide dismutase by Gdn·HCl. A dynamic fluorescence study. *Biochemistry 31*:7224–7230.

Milla ME, Sauer RT. 1994. P22 Arc repressor. Folding kinetics of a single-domain, dimeric protein. *Biochemistry 33*:1125–1133.

Miller S, Lesk AM, Janin J, Chothia C. 1987. The accessible surface area and stability of oligomeric proteins. *Nature 328*:834–836.

Monera OD, Shaw GS, Zhu BY, Sykes BD, Kay CM, Hodges RS. 1992. Role of interchain alpha-helical hydrophobic interactions in Ca^{2+} affinity, formation, and stability of a two-site domain in troponin C. *Protein Sci 1*:945–955.

Morjana NA, McKeone BJ, Gilbert HF. 1993. Guanidine hydrochloride stabilization of a partially unfolded intermediate during the reversible denaturation of protein disulfide isomerase. *Proc Natl Acad Sci USA 90*:2107–2111.

Mossing MC, Sauer RT. 1990. Stable, monomeric variants of λ Cro obtained by insertion of a designed β-hairpin sequence. *Science 250*:1712–1715.
Murphy KP, Freire E. 1992. Thermodynamics of structural stability and cooperative folding behavior in proteins. *Adv Protein Chem 43*:313–361.
Narhi LO, Rosenfeld R, Wen J, Arakawa T, Prestrelski SJ, Philo JS. 1993. Acid-induced unfolding of BDNF results in the formation of a monomeric "A state." *Biochemistry 32*:10819–10825.
Nicosia A, Monaci P, Tomei L, De Francesco R, Nuzzo M, Stunnenberg H, Cortese R. 1990. A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA binding structure of LFB1. *Cell 61*:1225–1236.
Pabo CO, Sauer RT, Sturtevant JM, Ptashne M. 1979. The λ repressor contains two domains. *Proc Natl Acad Sci USA 76*:1608–1612.
Pace CN. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol 131*:266–282.
Pace CN. 1990. Conformational stability of globular proteins. *Trends Biochem Sci 15*:14–17.
Pace CN, Laurents DV, Thomson JA. 1990. pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry 29*:2564–2572.
Pace CN, Tanford C. 1968. Thermodynamics of the unfolding of β-lactoglobin A in aqueous urea solutions between 5 and 55°. *Biochemistry 7*:198–208.
Pakula AA, Sauer RT. 1989. Amino acid substitutions that increase the thermal stability of the lambda Cro protein. *Proteins Struct Funct Genet 5*:202–210.
Peng X, Jonas J, Silva JL. 1993. Molten-globule conformation of Arc repressor monomers determined by high-pressure H NMR spectroscopy. *Proc Natl Acad Sci USA 90*:1776–1780.
Price N. 1992. Folding and assembly of multi-subunit proteins. In: Burgen A, Barnard EA, eds. *Receptor subunits and complexes*. Cambridge, UK: Cambridge University Press. pp 9–38.
Privalov PL. 1979. Stability of proteins: Small globular proteins. *Adv Protein Chem 33*:167–239.
Privalov PL, Gill SJ. 1988. Stability of protein structure and hydrophobic interaction. *Adv Protein Chem 39*:191–235.
Privalov PL, Griko YV, Venyaminov SY, Kutyshenko VP. 1986. Cold denaturation of myoglobin. *J Mol Biol 190*:487–498.
Privalov PL, Potekhin SA. 1986. Scanning microcalorimetry in studying temperature-induced changes in proteins. *Methods Enzymol 131*:4–51.
Raleigh DP, DeGrado WF. 1992. A de novo designed protein shows a thermally induced transition from a native to a molten globule-like state. *J Am Chem Soc 114*:10079–10081.
Raumann BE, Rould MA, Pabo CO, Sauer RT. 1994. DNA recognition by beta-sheets in the Arc repressor-operator crystal structure. *Nature 367*:754–757.
Reece LJ, Nichols R, Ogden RC, Howell EE. 1991. Construction of a synthetic gene for an R-plasmid-encoded dihydrofolate reductase and studies on the role of the N-terminus in the protein. *Biochemistry 30*:10895–10904.
Sacchetta P, Aceto A, Bucciarelli T, Dragani B, Santarone S, Allocati N, Di Ilio C. 1993. Multiphasic denaturation of glutathione transferase B1-1 by guanidinium chloride. Role of the dimeric structure on the flexibility of the active site. *Eur J Biochem 215*:741–745.
Sandberg WS, Terwilliger TC. 1993. Engineering multiple properties of a protein by combinatorial mutagenesis. *Proc Natl Acad Sci USA 90*:8367–8371.
Santoro MM, Bolen DW. 1992. A test of the linear extrapolation of unfolding free energy changes over an extended denaturant and concentration range. *Biochemistry 31*:4901–4907.
Schellman JA. 1978. Solvent denaturation. *Biopolymers 17*:1305–1322.
Silva JL, Silveira CF, Correia A Jr, Pontes L. 1992. Dissociation of a native dimer to a molten globule monomer. Effects of pressure and dilution on the association equilibrium of arc repressor. *J Mol Biol 223*:545–555.
Steif C, Weber P, Hinz HJ, Flossdorf J, Cesareni G, Kokkinidis M. 1993. Subunit interactions provide a significant contribution to the stability of the dimeric four-alpha-helical-bundle protein ROP. *Biochemistry 32*:3867–3876.
Tanford C. 1968. Protein denaturation. *Adv Protein Chem 23*:122–282.
Thompson KS, Vinson CR, Freire E. 1993. Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transcription factor GCN4. *Biochemistry 32*:5491–5496.
Timasheff SN. 1992. Water as ligand. Preferential binding and exclusion of denaturants in protein unfolding. *Biochemistry 31*:9857–9864.
Timm DE, deHaseth PL, Neet KE. 1994. Comparative equilibrium denaturation studies of the neurotrophins: NGF, BDNF, NT-3 and NT-4/5. *Biochemistry 33*:4667–4676.
Timm DE, Neet KE. 1992. Equilibrium denaturation studies of mouse beta-nerve growth. *Protein Sci 1*:236–244.
Zetina CR, Goldberg ME. 1980. Reversible unfolding of the b2 subunit of *Escherichia coli* tryptophan synthetase and its proteolytic fragments. *J Mol Biol 137*:401–414.
Zhang RG, Joachimiak A, Lawson CL, Schevitz RW, Otwinowski Z, Sigler PB. 1987. The crystal structure of *trp* aporepressor at 1.8 Å shows how binding tryptophan enhances DNA affinity. *Nature 327*:591–597.