FOR THE RECORD

Analysis of the stability of multimeric proteins by effective ΔG and effective m-values

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Abstract

Analyzing the stability of a multimeric protein is challenging because of the intrinsic difficulty in handling the mathematical model for the folded multimer-unfolded monomer equilibrium. To circumvent this problem, we introduce the concept of effective stability, ΔG_{eff} ($= -RT\ln K_{eff}$), where K_{eff} is the equilibrium constant expressed in monomer units. Analysis of the denaturant effect on ΔG_{eff} gives new insight into the stability of multimeric proteins. When a multimeric protein is mostly folded, the dependence of effective stability on denaturant concentration (effective ***m***-value) is simply the ***m***-value of its monomeric unit. However, when the protein is mostly unfolded, its stability depends on denaturant concentration with the ***m***-value of its multimeric form. We also find that the effective ***m***-value at the C_m is a good approximation of the apparent ***m***-value determined by fitting the equilibrium unfolding data from multimeric proteins with a two-state ***monomer*** model. Moreover, when the ***m***-value of a monomeric unit is estimated from its size, the effective stability of a multimeric protein can be determined simply from C_m and this estimated *m*-value. These simple and intuitive approaches will allow a facile analysis of the stability of multimeric proteins. These analyses are also applicable for high-throughput analysis of protein stability on a proteomic scale.

Keywords: protein stability; *m*-value; multimeric protein; oligomeric protein

Determining protein stability is the first step in investigating the thermodynamic properties of proteins. The only requirement is that the protein establishes a reversible equilibrium between the folded and unfolded states. Most proteins whose stabilities have been studied so far are monomeric. Despite the prevalence of multimeric proteins, stability determinations of multimeric proteins are quite rare, mostly because of the inherent difficulty in the treatment and analysis of their equilibrium behavior. Enhancing our knowledge of the stability of multimeric proteins requires the development of new and facile ways to analyze their equilibria.

The equilibrium between an *n*-mer protein and its unfolded monomer without any intermediate state[1] is shown in equation 1:

$$F_n \underset{\leftarrow}{\overset{\rightarrow}{}} nU, \qquad (1)$$

where the equilibrium constant of the reaction is

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[1] An alternative unfolding mechanism is also observed in multimeric protein unfolding in which dissociation into monomers and unfolding are not coupled:

$$F_n \underset{\leftarrow}{\overset{\rightarrow}{}} nF \underset{\leftarrow}{\overset{\rightarrow}{}} nU$$

The unfolding transition in this mechanism is nothing but an unfolding transition of a monomeric protein, which can be analyzed with conventional methods. Therefore, here we only focus on the mechanism shown in equation 1.

$$K_{unf} = \frac{[U]^n}{[F_n]}$$

The fraction of unfolded protein, f_D, is defined as in equation 2.

$$f_D = \frac{[U]}{P_t} \quad (2)$$

where P_t is the total protein concentration in monomer units. K_{unf} and ΔG° can be expressed as functions of f_D:

$$[F_n] = \frac{P_t (1 - f_D)}{n}$$

$$[U] = P_t f_D$$

$$K_{unf} = \frac{n f_D{}^n P_t{}^{n-1}}{1 - f_D} \quad (3)$$

$$\Delta G^\circ = -RT \ln \frac{n f_D{}^n P_t{}^{n-1}}{1 - f_D} \quad (4)$$

The challenge in studying multimeric proteins results from their reaction order. Even for multimeric proteins, ΔG° can be directly calculated from an experimentally determined f_D by equation 4 (Silinski et al. 2001). However, calculation of f_D from a given ΔG° is nontrivial, because equation 4 is n-th order. Solving the equation is feasible for monomeric and dimeric proteins. However, even for trimeric proteins, deriving a general expression of f_D as a function of K_{unf} is challenging (Backmann et al. 1998; Silinski et al. 2001; Güthe et al. 2004):

$$f_D = \sqrt[3]{\frac{1}{2}c + \frac{\sqrt{3}}{18}c\sqrt{4c+27}} - \frac{c}{\sqrt[3]{\frac{1}{2}c + \frac{\sqrt{3}}{18}c\sqrt{4c+27}}}, \text{ where } c = \frac{K_{unf}}{3P_t^2}$$

For $n > 3$, the equation needs to be solved numerically (Johnson et al. 1995; Boudker et al. 1997; Panse et al. 2000). This problem precludes the development of a general description for the stability of multimeric proteins. Moreover, since K_{unf} is not unitless for multimeric proteins, it is much less intuitive and less useful for comparative purposes than is K_{unf} for monomeric proteins.

The effective stability

Is there a more intuitive approach similar to that used for monomeric proteins to understand the thermodynamic stability of multimeric proteins? To address this question, we have developed an approach using an effective ΔG (ΔG_{eff}), instead of ΔG° for the standard condition:

$$\Delta G_{eff} = -RT \ln K_{eff} = -RT \ln \frac{f_D}{1 - f_D}, \quad (5)$$

where f_D is defined as in equation 2. K_{eff} is simply the ratio of unfolded protein concentration to folded protein concentration in monomer units. ΔG_{eff} and K_{eff} are pseudo-thermodynamic parameters where the actual order of chemical equation is ignored. Unlike ΔG°, ΔG_{eff} and K_{eff} are dependent on the total protein concentration of the system. However, these pseudo-thermodynamic parameters are more intuitive in that they relate directly to the amount of folded protein in the system. For example, ΔG_{eff} is always zero when half of protein is unfolded, that is, $f_D = 0.5$, while ΔG° of a multimeric protein has a nonzero value when $f_D = 0.5$.

Next, we derive a general equation for $\Delta G_{eff}(H_2O)$. As mentioned earlier, analytical derivation of $\Delta G_{eff}(H_2O)$ from $\Delta G^\circ(H_2O)$ in an n-mer system is mathematically impossible. If one assumes that in water f_D will be very small (a reasonable assumption for a stable protein under aqueous condition), then $\Delta G^\circ(H_2O)$ can be directly related to $\Delta G_{eff}(H_2O)$. From equations 4 and 5,

$$\Delta G^\circ(H_2O) = -RT \ln(n f_D{}^n P_t{}^{n-1}) = -nRT \ln f_D - RT \ln(n P_t{}^{n-1})$$

$$\Delta G_{eff}(H_2O) = -RT \ln f_D$$

Therefore,

$$\Delta G_{eff}(H_2O) = \frac{\Delta G^\circ(H_2O)}{n} + \frac{RT}{n} \ln(n P_t{}^{n-1}) \quad (6)$$

Here, $\Delta G^\circ/n$ is the ΔG° per monomer unit (Boudker et al. 1997), which is concentration-independent and an intrinsic property of the protein. The second term, however, is not protein-specific; it depends only on the total protein concentration and the number of monomer units in the multimer. This second term is identical for any n-meric protein under identical total protein concentration. Therefore, ΔG_{eff} can be dissected into two terms: a protein-specific term, and a concentration-dependent term.

The separate terms in equation 6 allow a facile comparison of protein stability across proteins with different oligomeric states, which is not feasible with ΔG°. Table 1 shows

Table 1. *Comparison of* ΔG°, ΔG°/*n and* ΔG_{eff} *of various multimeric proteins*

Proteins	Oligomeric state	$\Delta G°$ (kcal/mole of *n*-mer)	$\Delta G°/n$ (kcal/mole of monomer)	ΔG_{eff} (kcal/mole of monomer)
Trp aporepressor	2	23.3 ± 0.9[a]	11.7 ± 0.5	9.1 ± 0.5
HIV-1 protease	2	14.2 ± 1.4[b]	7.1 ± 0.7	4.6 ± 0.7
Arc repressor	2	11[c]	5.5	3.0
Adenylate kinase	3	30.5[d]	10.2	6.8
T4 fibritin	3	21.3 ± 0.1[e]	$7.1 \pm 0.0_3$	$3.7 \pm 0.0_3$
p53	4	29.5	7.4[h]	3.5
SecB	4	28 ± 2[f]	7.0 ± 0.5	3.1 ± 0.5
4-Oxalocrotonate tautomerase	6	68 ± 3[g]	11.3 ± 0.5	7.0 ± 0.5
GroES	7	63	9.0[i]	4.5

The $\Delta G°$ value for each protein was collected from the literature. For p53 and GroES, $\Delta G°$ values were calculated from the $\Delta G°/n$ reported in the literature. The ΔG_{eff} value of each protein was calculated with equation 6, with a protein concentration of 100 μM in monomer units.
[a] Gittelman and Matthews 1990; [b] Grant et al. 1992; [c] Bowie and Sauer 1989; [d] Backmann et al. 1998; [e] Güthe et al. 2004; [f] Panse et al. 2000; [g] Silinski et al. 2001; [h] Johnson et al. 1995; [i] Boudker et al. 1997.

$\Delta G°/n$ extracted from experimental data available for several multimeric proteins. While $\Delta G°$ values vary significantly, $\Delta G°/n$ values are all between 5 kcal/mole and 12 kcal/mole, which is also the usual range for monomeric protein stability. The ΔG_{eff} values at 100 μM monomer concentration were also calculated for the same proteins (Table 1). When two proteins in different oligomeric states have the same $\Delta G°/n$ values, the second term of the equation 6 explains the difference in ΔG_{eff}, which is directly related to the unfavorable oligomerization at a nonstandard concentration. For example, $\Delta G°/n$ of Trp aporepressor and 4-oxalocrotonate tautomerase (4-OT) are quite similar: 11.7 kcal/mole and 11.3 kcal/mole, respectively (Table 1). However, because Trp aporepressor is a dimer and 4-OT is a hexamer, folding of 4-OT involves a bigger loss of entropy than does Trp aporepressor. When the two proteins are at the same concentration, the effective stabilities of 4-OT and Trp aporepressor differ mostly in the second term of equation 6 (2.1 kcal/mole at 100 μM in monomer units).

$\Delta G°/n$ values can easily be used to analyze the effect of mutations on the stability of multimeric proteins, because mutations do not affect the free energy change of the second term in equation 6. Therefore, the effect of mutations on ΔG_{eff} is described simply as:

$$\Delta\Delta G_{eff}(H_2O) = \frac{\Delta\Delta G°(H_2O)}{n},$$

which shows that the mutational effect on $\Delta G°/n$ reflects directly the change in ΔG_{eff} at any protein concentration. This relationship also supports the utility of ΔG_{eff} and $\Delta G°/n$ in stability analysis of multimeric proteins.

The effect of denaturant on ΔG_{eff}

The simplest method for determining protein stability is by monitoring chemical-induced denaturation profiles, combined with a two-state approximation and linear extrapolation (Greene and Pace 1974).

$$\Delta G° = \Delta G°(H_2O) + mD, \tag{7}$$

where D is the denaturant concentration and m is the dependence of the stability on denaturant. Unfolding equilibrium constants, K_{unf}, can be measured directly in the transition zone, where detectable amounts of folded and unfolded proteins are in equilibrium. Then, $\Delta G°(H_2O)$ can be determined by extrapolating $\Delta G°$ values ($= RT\ln K_{unf}$) from varying concentrations of denaturant.

To show how denaturant affects ΔG_{eff} of multimeric proteins, we define m_{eff} as:

$$m_{eff} = \frac{\partial \Delta G_{eff}}{\partial D} \tag{8}$$

By using equation 5,

$$m_{eff} = \frac{\partial \Delta G_{eff}}{\partial D} = -RT\frac{1-f_D}{f_D}\frac{\partial}{\partial D}\left(\frac{f_D}{1-f_D}\right) = -\frac{RT}{f_D(1-f_D)}\frac{\partial f_D}{\partial D} \tag{9}$$

$\partial f_D/\partial D$ can be derived by using equations 4 and 7. The two equations are combined to yield:

$$-RT\ln\frac{n f_D{}^n P_t{}^{n-1}}{1-f_D} = \Delta G°(H_2O) + mD \tag{10}$$

By differentiating equation 10 with respect to D,

$$-RT\frac{\partial}{\partial D}(\ln n P_t{}^{n-1} + n\ln f_D - \ln(1-f_D)) = m$$

$$\frac{n}{f_D}\frac{\partial f_D}{\partial D}+\frac{1}{1-f_D}\frac{\partial f_D}{\partial D}=-\frac{m}{RT}$$

$$\frac{\partial f_D}{\partial D}=-\frac{m}{RT}\frac{f_D(1-f_D)}{n+(1-n)f_D} \quad (11)$$

From equations 9 and 11,

$$m_{eff}=\frac{m}{n+(1-n)f_D} \quad (12)$$

This simple equation clearly shows the denaturant dependence of multimeric proteins. For a monomeric protein ($n = 1$), m_{eff} is equal to m and constant with respect to denaturant. For multimeric proteins ($n > 1$), m_{eff} is not a constant. When the protein is mostly folded ($f_D \sim 0$), m_{eff} is close to m/n. When the protein is mostly unfolded ($f_D \sim 1$), m_{eff} is close to m.

Interestingly, m/n is approximately the expected m-value for a monomeric unit of an n-mer. Based on a study of reported m-values, Myers et al. demonstrated that m-values show a strong correlation with the size or the number of amino acids in a protein (Myers et al. 1995). It follows that the expected m-value for a hypothetical folded monomeric unit of an n-mer would be $1/n$ of the value of the n-mer protein (m/n). Therefore, when the protein is mostly folded, m_{eff} is approximately the expected m-value of the monomeric unit.

This denaturant-dependent behavior of m_{eff} is shown clearly in Figure 1. In this simulation, ΔG_{eff} of each multimeric protein was determined numerically using equations 4 and 5. All the proteins were given the same expected m-value for a monomeric unit of 2 kcal/(mole·M) ($m/n = 2$). For ease of comparison, all proteins were assigned to have the same C_m value (the denaturant concentration where $f_D = 0.5$) of 3 M. As shown in Figure 1, under native condition ($D < C_m$), the denaturant dependence of ΔG_{eff} shows a slope of 2, regardless of the oligomeric state. As predicted by equation 12, this slope corresponds to the expected m-value of the monomeric unit. Under denaturing conditions ($D > C_m$); however, the slope of the denaturant dependence for ΔG_{eff} corresponds to the m-value of each oligomeric protein.

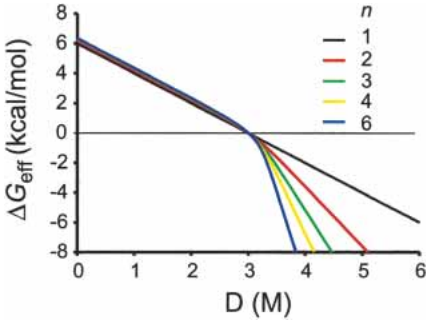

Figure 1. The effect of denaturant on effective ΔG of multimeric proteins. n represents the oligomeric state of each protein. The m/n values and C_m of all the proteins were set to be 2 kcal/(mole·M) and 3 M, respectively, for comparison. $\Delta G°(H_2O)$ was determined with equation 14 by using these parameters. The fraction of unfolded protein, f_D, was determined from $\Delta G°$ by solving equation 4 numerically. ΔG_{eff} was calculated with this f_D by equation 5. Total protein concentration was set to be 2 μM in monomer units. However, the result is indifferent to total protein concentration, because C_m was fixed.

The behavior predicted by equation 12 agrees with published data using hydrogen exchange and mass spectrometry to evaluate the stability of multimeric proteins (Powell et al. 2002). When this technique, called SUPREX (Ghaemmaghami et al. 2000), was applied for multimeric proteins, the observed m-values were noted to be $1/n$-th of the m-value expected for the multimeric proteins. These m-values were derived at the C_m^{SUPREX}, which is lower than the actual C_m because of the kinetics of hydrogen exchange (Ghaemmaghami et al. 2000):

$$C_m^{SUPREX}=C_m-\frac{RT}{m}\ln\left(\frac{<k_{int}>t}{0.693}-1\right),$$

which suggests that f_D is close to 1 at C_m^{SUPREX}. Therefore, m_{eff} determined by SUPREX is m_{eff} at $f_D \sim 1$, which is close to m/n as shown in equation 12.

Analysis of denaturation data from multimeric proteins

The simplest treatment of chemically induced denaturation data from multimeric proteins would be to use the simple two-state monomer model. What m-values would result from this curve fitting? Even though this approach clearly uses an incorrect model, the curve fitting would yield the correct C_m, because this parameter is model-free. Moreover, only a limited range of ΔG_{eff} can be determined by experiment—those in the transition zone where D is near C_m. Therefore, experimental ΔG_{eff} values are unlikely to show the biphasic patterns shown in Figure 1. Rather, experimentally determined plots of ΔG_{eff} versus D would show a more or less linear tendency unless n is very large. The m-value from the simplified curve-fitting with a monomer model would be close to the m_{eff} at C_m. From equation 12, m_{eff} at C_m can be calculated by setting $f_D = 0.5$:

$$m_{eff}(C_m)=\frac{2}{n+1}m \quad (13)$$

Table 2 shows that indeed the m-values obtained by this incorrect, yet simple, monomer model correlate extremely well with the expected m_{eff} at the midpoint determined by the above equation. The m-values in Table 2 were deter-

Table 2. *Comparison of* m_{eff} *at* C_m *with* m*-values determined with a two-state monomer model*

Oligomeric state	*m*-value used for generating simulation data	m_{eff} at C_m	*m*-value from curve fitting with a monomer model
1	−2.0	−2.0	−2.0 ± 0.0
2	−4.0	−2.7	−2.7 ± 0.1
3	−6.0	−3.0	−3.1 ± 0.1
4	−8.0	−3.2	−3.3 ± 0.1
6	−12.0	−3.4	−3.5 ± 0.2

The fractions of unfolded protein, f_D, were calculated by assuming $m/n = -2$ kcal/(mole · M) and $C_m = 3$ M for imaginary *n*-mer proteins (Fig. 1). These f_D values were fit with a two-state monomer model to determine *m*-values. The m_{eff} at C_m were also calculated with equation 13 from the *m*-values used to calculate f_D. The unit of *m*-values is kcal/(mole · M).

mined by fitting the f_D values used to generate Figure 1 with a two-state monomer model.

The above analysis demonstrates that the *m*-value of multimeric proteins can be reasonably determined by applying a two-state monomer model and calculating m_{eff} at the midpoint. This monomer model does a better job of approximating m_{eff} at the C_m when *n* is smaller. Because m_{eff} is not actually constant, one caveat to this approach is that the curve-fitting will depend on the distribution of data points in the transition zone. Introducing more data points on the unfolded side of the transition ($0.5 < f_D < 1$) results in *m*-values bigger than m_{eff} at C_m. It is also important to stress that the stabilities that result from this curve fitting with the monomer model are meaningless, because the slope is treated as a constant, yet m_{eff} depends on denaturant.

Determination of ΔG_{eff} *from* C_m

Can $\Delta G_{eff}(H_2O)$ be determined simply by knowing the midpoint? As mentioned earlier, with the statistical data from the study by Myers et al. (1995), one can estimate *m*-values simply from the size of proteins. In monomeric proteins, ΔG_{unf} can be reasonably approximated by multiplying the estimated *m*-value and C_m. Is this approach valid for multimeric proteins? The simulated data in Figure 1 suggest that ΔG_{eff} in H_2O might be approximated by C_m and m/n, the *m*-value for monomeric unit, regardless of the oligomeric state of a protein.

To verify this observation, we derive an equation for calculating ΔG_{eff} from the C_m and *m*-value. In multimeric proteins, $\Delta G°$ is not zero at the C_m. Rather, according to equation 4, $\Delta G°$ at the C_m is expressed as (Backmann et al. 1998; Ragone 2000):

$$\Delta G°(C_m) = -RT \ln n\left(\frac{P_t}{2}\right)^{n-1}$$

By using equation 7,

$$\Delta G°(C_m) = \Delta G°(H_2O) + mC_m = -RT \ln n\left(\frac{P_t}{2}\right)^{n-1}$$

$$\Delta G°(H_2O) = -mC_m - RT \ln n\left(\frac{P_t}{2}\right)^{n-1} \quad (14)$$

By using equations 6 and 14, ΔG_{eff} can be expressed in a simple equation:

$$\Delta G_{eff}(H_2O) = -\frac{m}{n} C_m + R(n), \text{ where } R(n) = \frac{n-1}{n} RT \ln 2 \quad (15)$$

$R(n)$ is a residual function of *n*. This function is responsible for the slight differences in $\Delta G_{eff}(H_2O)$ values between different oligomers in Figure 1. For monomeric proteins, $R(n)$ is zero. In all cases, $R(n)$ is smaller than $RT\ln 2$ (~0.41 kcal/mole at 25°C), which is quite negligible in most practical applications, and as shown in Figure 1. Because m/n is simply the *m*-value of a monomeric unit of the *n*-mer, $\Delta G_{eff}(H_2O)$ can be deduced from two parameters: the *m*-value of a monomeric unit calculated from the statistical data by Myers et al. and the experimentally determined C_m. Surprisingly, knowing the number of monomers in the multimer is not necessary to determine $\Delta G_{eff}(H_2O)$, because the two parameters, the *m*-value of the monomeric unit (m/n) and the C_m, are model-free.

Conclusions

We show here that the use of effective ΔG and effective *m*-value provides valuable information about the stabilities of multimeric proteins. ΔG_{eff} is a useful parameter that gives an intuitive appreciation of the stability of multimeric proteins; one can calculate the fraction of unfolded protein from ΔG_{eff} of a multimeric protein as easily as doing so from the $\Delta G°$ of a monomeric protein. To understand the effect of denaturant on the stability of multimeric protein, we define m_{eff} as the derivative of ΔG_{eff} with respect to denaturant concentration. Analysis of m_{eff} shows that denaturant affects ΔG_{eff} in a biphasic way. When the *m*-value of an *n*-mer protein is *m*, m_{eff} of this protein is m/n under folding conditions and *m* under denaturing conditions. Thus, although direct analysis of $\Delta G°$ is not feasible for multimeric proteins, ΔG_{eff} and m_{eff} provide a facile alternative way to compare the stability of these proteins. Additionally, ΔG_{eff} can be determined without prior knowledge of the oligomeric state of a protein. This unique property of ΔG_{eff} could facilitate high-throughput analysis of protein stability on a proteomic scale.

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