Ligand binding and thermodynamic stability of a multidomain protein, calmodulin

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Abstract

Chemical and thermal denaturation of calmodulin has been monitored spectroscopically to determine the stability for the intact protein and its two isolated domains as a function of binding of Ca^{2+} or Mg^{2+} . The reversible urea unfolding of either isolated apo-domain follows a two-state mechanism with relatively low ΔG_{20}° values of ~ 2.7 (N-domain) and ~ 1.9 kcal/mol (C-domain). The apo-C-domain is significantly unfolded at normal temperatures (20–25 °C). The greater affinity of the C-domain for Ca^{2+} causes it to be more stable than the N-domain at $[\text{Ca}^{2+}] \geq 0.3$ mM. By contrast, Mg^{2+} causes a greater stabilization of the N- rather than the C-domain, consistent with measured Mg^{2+} affinities.

For the intact protein ($\pm \text{Ca}^{2+}$), the bimodal denaturation profiles can be analyzed to give two ΔG_{20}° values, which differ significantly from those of the isolated domains, with one domain being less stable and one domain more stable. The observed stability of the domains is strongly dependent on solution conditions such as ionic strength, as well as specific effects due to metal ion binding. In the intact protein, different folding intermediates are observed, depending on the ionic composition. The results illustrate that a protein of low intrinsic stability is liable to major perturbation of its unfolding properties by environmental conditions and liganding processes and, by extension, mutation. Hence, the observed stability of an isolated domain may differ significantly from the stability of the same structure in a multidomain protein. These results address questions involved in manipulating the stability of a protein or its domains by site directed mutagenesis and protein engineering.

Keywords: calcium binding; calmodulin; chemical denaturation; circular dichroism; thermal denaturation

Studies of the thermodynamic stability of individual proteins have been greatly stimulated by the availability of recombinant proteins, and their corresponding mutants with specifically modified residues. Advances in theoretical understanding of protein stability (Schellman, 1987; Privalov, 1992; Shortle, 1992) have provided the foundation for a consistent interpretation of a wide variety of equilibrium and kinetic phenomena associated with protein folding and unfolding. Experimental approaches involve spectroscopic, hydrodynamic, and calorimetric methods applied to chemical and thermal denaturation. Such studies have been extensively reviewed (Creighton, 1992). In this work, we investigate the thermodynamic stability of calmodulin (CaM), a two-domain protein with important Ca^{2+} binding functions. These physical properties relate to

specific questions of the stability of CaM in vivo, and the independence of the CaM domains, as well as more general questions of the relationship between stability of a subdomain relative to that of the whole protein, and the thermodynamic coupling of ligand binding and stability. CaM therefore presents an original and challenging system for protein folding studies and illustrates general features important for protein stability and design.

CaM is a highly conserved, eukaryotic calcium binding protein, which mediates a variety of physiological processes by interacting with high affinity to activate a number of different enzymes. The structure of CaM and some target peptide complexes have been solved to high resolution by X-ray crystallography and NMR spectroscopy (Crivici & Ikura, 1995, and references therein). CaM itself consists of two structurally similar globular domains, connected by a flexible linker, which allows the two domains to adopt different positions with respect to each other and to bind targets. Each domain contains a pair of helix-loop-helix calcium-binding motifs (EF-hands), linked by a short region of antiparallel β -sheet. The Ca^{2+} affinity for sites III and IV in the C-terminal domain is higher than for sites I and II in the N-terminal domain and positive cooperativity is observed between sites within a domain (Linse et al., 1991; Martin et al., 1996).

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Abbreviations: CaM, *Drosophila melanogaster* calmodulin; CD, circular dichroism; HPLC, high-performance liquid chromatography; LEM, linear extrapolation model; TrIC and Tr2C, N- and C-terminal tryptic fragments of calmodulin; 5,5'-dibromo BAPTA, 5,5'-dibromo-1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; 5-nitro BAPTA, 5-nitro-1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; UV, ultraviolet.

CaM can be cleaved proteolytically with trypsin into two closely homologous domains (48% sequence identity, 69% similarity) with similar secondary structure and conformation. Although the domains contain some common determinants for interactions with target molecules, they can make distinct contributions to target binding and activation of enzymes (Newton et al., 1984; Persechini et al., 1994; Bayley et al., 1996; Barth et al., 1998). The two domains of CaM are generally considered to behave as independent units, since the optical spectroscopic (Martin & Bayley, 1986), kinetic (Martin et al., 1985; Suko et al., 1986), and calcium binding (Linse et al., 1991; Martin et al., 1996) properties of intact CaM are well represented by a summation of those of its tryptic fragments. However, evidence that the presence of one domain does affect the calcium-binding properties of the other has been reported, both for CaM (Sorensen & Shea, 1998) and for the related protein, troponin C (Moncrieffe et al., 1999).

Previous investigations on the stability of CaM and its fragments, performed with optical spectroscopy and/or microcalorimetry showed that apo-CaM is much less stable than holo-CaM (Brzeska et al., 1983; Tsalkova & Privalov, 1985; Martin & Bayley, 1986). Several studies have concluded that the apo N-domain is more stable than the apo C-domain both when isolated (Brzeska et al., 1983; Sorensen & Shea, 1998) and in the intact protein (Browne et al., 1997; Protasevich et al., 1997).

We report here the results of a systematic study of the thermodynamic stability of CaM and its constituent domains as a function of binding of Ca^{2+} (or Mg^{2+}), using thermal and chemical denaturation, and spectroscopic monitoring using far-UV CD and tyrosine fluorescence and absorption (cf. Masino et al., 1999). The results show the distinctive effects of urea and GuHCl in their interactions with CaM. Using urea as denaturant, the stability (ΔG_{20}°) of the apo form of the two domains in CaM can be accurately determined. This permits the quantitative analysis of the complex differential sensitivity of stability of the holo-domains, which is due to coupling of the intrinsic stability of the individual domains to their ligand binding properties. Given the low thermodynamic stability of either apo-domain, the stability of the domains of CaM is strongly influenced by general environmental factors as well as specific ligand interactions. These properties are of general significance to the folding properties and engineering of mutants of multidomain proteins and to the conformational properties of other proteins of marginal or low thermodynamic stability. They also suggest ways in which the relative stabilities of different domains can be manipulated experimentally by solution conditions, properties that could be exploited in the design of proteins whose stability can be controlled by external factors.

Results

Chemical unfolding of apo-proteins

Figure 1A shows typical far-UV CD data for urea-induced denaturation of apo-CaM, apo-Tr1C, and apo-Tr2C. Table 1 summarizes the results of the LEM analyses. The results for the fragments show that apo-Tr1C is marginally more stable than apo-Tr2C under these buffer conditions. The curve for intact apo-CaM clearly shows two resolvable transitions, which are denoted as CaM-1 and CaM-2 in Table 1. The denaturant m values determined for the two transitions were very similar to those obtained for the isolated fragments, confirming that the two transitions correspond to unfolding of individual CaM domains. The parameters obtained from the

Fig. 1. Chemical denaturation of apo-proteins. (A) Urea- and (B) GuHCl-induced denaturation of apo-Tr1C (Δ), apo-Tr2C ($\square$), and intact apo-CaM ($\circ$). Unfolding was monitored at 20°C using far-UV CD. The buffer was 25 mM Tris, 100 mM KCl, pH 8. The solid lines are the computed best fits. The dotted line in each panel represents the sum of the curves for Tr1C and Tr2C.

analysis of fluorescence and absorption data (monitoring Tyr138 in the C-domain, Table 1) identify the CaM-1 transition in intact apo-CaM with the C-domain, and hence CaM-2 with the N-domain. This assignment is consistent with the result that apo-Tr1C is more stable than apo-Tr2C (Table 1). Figure 1A shows that the unfolding curve for intact apo-CaM differs from the sum of the unfolding curves for the isolated fragments. The analyses (Table 1) show that the C-domain in apo-CaM is destabilized by ~ 0.45 kcal/mol compared to free apo-Tr2C, while the N-domain in apo-CaM is stabilized by ~ 0.8 kcal/mol compared to free apo-Tr1C.

The pattern of behavior observed with GuHCl (Fig. 1B, Table 1) is similar to that observed with urea. The unfolding curve for intact apo-CaM again differs from the sum of the unfolding curves for the isolated fragments (see Fig. 1B), and the results of the analyses again show that the N-domain of apo-CaM is more stable than apo-Tr1C, while the C-domain is less stable than apo-Tr2C. However, the ΔG_{20}° values determined with GuHCl are consistently larger than those obtained with urea. The possible reasons for this observation are discussed below.

Thermal unfolding of apo-proteins

Figure 2A shows the reversible thermal unfolding curves for apo-CaM and its isolated domains monitored by far-UV CD. The pa-

Table 1. Urea and GuHCl unfolding of apo-proteins (25 mM Tris, 100 mM KCl, pH 8, 20 °C)

Transition	Denaturant	Method ^a	ΔG_{20}° ^b (kcal/mol)	$[D]_{1/2}$ (M)
Tr1C	Urea	CD	2.73	3.1
Tr2C	Urea	CD	1.91	2.17
Tr2C	Urea	Flu	1.85	2.1
CaM-1 ^c	Urea	CD	1.45	1.65
CaM-2 ^c	Urea	CD	3.55	4.03
CaM-C ^c	Urea	Flu	1.52	1.73
CaM-C	Urea	Abs	1.50	1.71
Tr1C	GuHCl	CD	3.77	2.04
Tr2C	GuHCl	CD	2.65	1.43
CaM-1	GuHCl	CD	2.41	1.3
CaM-2	GuHCl	CD	4.35	2.35
CaM-2	GuHCl	CD	4.35	2.35
CaM-C	GuHCl	Flu	2.31	1.25
CaM-C	GuHCl	Abs	2.37	1.28

^aCD, Flu, and Abs identify unfolding monitored using far-UV CD (220 nm), Tyr138 fluorescence (320 nm), and Tyr138 absorption (288 nm).

^b ΔG_{20}° values were calculated as $-[D]_{1/2} \times m_{av}$, with $m_{av} = -0.88$ kcal/mol M (urea) and -1.85 kcal/mol M (GuHCl) (see Materials and methods).

^cCaM-1 and CaM-2 indicate the first and second transitions resolved for intact CaM (far-UV CD), assigned to the C- and N-domains, respectively (see text). CaM-C represents unfolding of the C-domain in intact CaM (absorption and fluorescence of Tyr138).

rameters obtained from the analyses are in Table 2. As for chemical unfolding, apo-Tr1C is more stable than apo-Tr2C and the curve for intact CaM differs from the sum of the curves of the tryptic fragments. Table 2 shows that the C-domain in apo-CaM is less stable than the isolated apo-Tr2C by ~ 0.8 kcal/mol, while the N-domain in apo-CaM is more stable than apo-Tr1C by ~ 0.5 kcal/mol. Other workers (Tsalkova & Privalov, 1985; Sorensen & Shea, 1998) have reached similar conclusions about differences in thermal stability between isolated fragments and the corresponding domains in intact apo-CaM. The values for T_m and ΔH_m obtained for the isolated fragments and for the domains in intact CaM are in good agreement with those measured by Sorensen and Shea (1998) under slightly different solution conditions (T_m and ΔH_m of 49.73 °C, 45.57 kcal/mol for Tr1C, 49.89 °C, 34.67 kcal/mol for Tr2C, 47.35 °C, 28.14 kcal/mol for the CaM-1 transition, and 59.95 °C and 44.17 kcal/mol for the CaM-2 transition). The thermal unfolding of the C-domain in intact apo-CaM has also been monitored using Tyr138 fluorescence. The results (Fig. 2B; Table 3 (100 mM KCl)), confirm the observation about the relative stabilities of apo-Tr2C and the C-domain in apo-CaM. The values of ΔG_{20}° obtained for apo-CaM and its domains from thermal denaturation (Tables 2, 3) agree slightly better with the values determined using urea as a denaturant, compared with those determined using GuHCl (Table 1). Calculations of the ΔG° values at 37 °C (in 100 mM KCl, pH 8) show that the degree of unfolding of the C-domain in intact apo-CaM is significant ($\sim 24\%$), while that of the N-domain is much less ($<2\%$) (Table 2).

Effect of ionic strength and comparison between urea and GuHCl

The effect of ionic strength on the stability of the apo proteins has been studied by measuring free energies of unfolding at different

Fig. 2. Thermal denaturation of apo-proteins. **A:** Thermal denaturation of apo-Tr1C (Δ), apo-Tr2C ($\square$), and intact apo-CaM ($\circ$) monitored using far-UV CD. The buffer was 25 mM Tris, 100 mM KCl, pH 8. The dotted line represents the sum of the curves for Tr1C and Tr2C. **B:** Thermal denaturation of apo-CaM monitored using Tyr138 fluorescence. The buffer was 10 mM Hepes, 100 mM KCl, pH 8. The solid lines are the computed best fits.

[KCl]. The results in Table 3 (thermal unfolding of apo-CaM monitored using Tyr138 fluorescence) and Table 4 (urea-induced unfolding monitored by far-UV CD) show that increasing the ionic strength increases the stability of the proteins. The sensitivity of the intensity of the far-UV CD spectrum of apo-CaM to experimental conditions was previously noted (Martin & Bayley, 1986).

The observation that the free energies of unfolding determined with GuHCl are consistently larger than those obtained with urea (Table 1) suggests that the apo proteins may be stabilized by low (nondenaturing) concentrations of GuHCl. Tyr138 fluorescence measurements of thermal unfolding of the C-domain of apo-CaM (Table 3) show that small concentrations of GuHCl ($= 0.2$ M) have a stabilizing effect similar to that of KCl. Similar behavior, with maximal effect at 0.3 M GuHCl, has been reported by Fredricksen and Swenson (1996) in experiments with residues 1–85 of chicken TnC. GuHCl (unlike urea) is positively charged, and therefore causes changes in the ionic strength of the solution. This suggests that the unfolding parameters for CaM determined using urea are more appropriate for thermodynamic analysis (cf. Monera et al., 1994).

Table 2. Thermal unfolding of apo-proteins (far-UV CD; 25 mM Tris, 100 mM KCl, pH 8)

Transition	T_m (°C)	ΔH_m (kcal/mol)	ΔG_{20}° ^a (kcal/mol)	% Unfolded (20 °C)	ΔG_{37}° ^a (kcal/mol)	% Unfolded (37 °C)
Tr1C	50.3	45.6	3.21	0.4	1.67	6.2
Tr2C	49.4	36.5	2.22	2.2	1.21	12.3
CaM-1 ^b	45.7	29.1	1.43	7.1	0.69	24.3
CaM-2 ^b	59.5	46.1	3.69	0.2	2.55	1.5

^a Values of ΔG° were calculated using ΔC_p values of 0.727 kcal/(K mol) (apo-Tr1C and CaM-2) and 0.804 kcal/(K mol) (apo-Tr2C and CaM-1) (Sorensen & Shea, 1998).

^b CaM-1 and CaM-2 indicate the first and second transitions resolved for intact CaM, assigned to the C- and N-domains, respectively (see text).

Chemical unfolding of Ca-saturated proteins

The calcium saturated (holo) forms of CaM and its domains are substantially more stable than the apo-forms, as predicted by the theory of ligand binding (see Materials and methods). Figure 3 shows typical far-UV CD data for the denaturation of the holo-forms of CaM, Tr1C, and Tr2C induced by urea in the presence of 0.3 mM calcium (Fig. 3A) and by GuHCl in the presence of 1.0 mM calcium (Fig. 3B). Table 5 summarizes the results of the LEM analyses. The binding of calcium causes a reversal of the relative stability of the isolated holo-domains compared to their apo-forms, so that at $[Ca^{2+}] = 0.3$ mM, holo-Tr2C is more stable than holo-Tr1C. This is consistent with Tr2C binding calcium more strongly than Tr1C; the ligand interaction free energy is therefore higher for Tr2C (Equation 5).

In the case of GuHCl, the assignment of the CaM-1 and CaM-2 transitions is unambiguous. The relative stabilities of the isolated fragments and the comparison of the far-UV CD and fluorescence data identifies the CaM-2 transition with the C-domain. However, the midpoint and ΔG_{20}° values determined from fluorescence data are somewhat higher than those determined from the CD data. Nevertheless, it is clear that the order of domain stabilities observed in holo-CaM is reversed compared to that in apo-CaM, with the C-domain now more stable than the N-domain. This is again consistent with the greater affinity of sites III and IV (C-domain) for calcium. Figure 3B shows that the unfolding curve for intact holo-CaM differs from the sum of the unfolding curves for the fragments. The C-domain in holo-CaM is stabilized compared to

free holo-Tr2C while the N-domain in holo-CaM is destabilized compared to free holo-Tr1C (Table 5). This is the opposite of the situation observed with intact apo-CaM: in both cases (apo- and holo-protein) the less stable domain in the intact protein is destabilized relative to its properties as an isolated domain.

In the case of urea-induced denaturation, it is also clear that the unfolding curve for intact holo-CaM (0.3 mM Ca^{2+}) differs from the sum of the unfolding curves for the fragments (Fig. 3A). However, the assignment of the CaM-1 and CaM-2 transitions is not unambiguous with urea because the holo-domains show very similar stability at this $[Ca^{2+}]$. Comparison with the fluorescence results suggests that the CaM-2 transition again corresponds to unfolding of the C-domain; i.e., as with GuHCl, the C-domain is stabilized compared to holo-Tr2C, while the N-domain is destabilized compared to holo-Tr1C.

Stabilization by calcium

To quantitate further the stabilization conferred by calcium binding, the far-UV CD unfolding profiles were measured at different

Table 3. Effects of KCl on the thermal unfolding of the apo-CaM C-domain (Tyr138 fluorescence; 10 mM Hepes, pH 7.5)

Salt	T_m (°C)	ΔH_m (kcal/mol)	ΔG_{20}° ^a (kcal/mol)	% Unfolded (20 °C)
None	31.3	25.3	0.81	21
0.1 M KCl	42.8	31.5	1.59	6.0
0.2 M KCl	47.3	33.5	1.89	3.7
0.1 M GuHCl	42.7	32.9	1.69	5.2
0.2 M GuHCl	44.9	32.3	1.75	4.8

^a Values of ΔG_{20}° were calculated using $\Delta C_p = 0.804$ kcal/(K mol) (Sorensen & Shea, 1998).

Table 4. Effects of KCl on urea unfolding of apo-proteins (far-UV CD; 25 mM Tris, pH 8.0, 20 °C)

Transition	[KCl] (M)	ΔG_{20}° ^a (kcal/mol)	$[D]_{1/2}$ (M)
Tr1C	<5	2.06	2.34
	30	2.44	2.77
	100	2.73	3.1
Tr2C	<5	1.15	1.31
	30	1.49	1.69
	100	1.91	2.17
CaM-1 ^b	<5	~0.5	~0.6
CaM-2 ^b	<5	2.63	2.99
CaM-1	100	1.45	1.65
CaM-2	100	3.55	4.03
CaM-1	200	1.82	2.07
CaM-2	200	3.80	4.32

^a ΔG_{20}° values were calculated as $-[D]_{1/2} \times m_{av}$, with $m_{av} = -0.88$ (see Materials and methods).

^b CaM-1 and CaM-2 indicate the first and second transitions resolved for intact CaM, assigned to the C- and N-domains, respectively (see text).

Fig. 3. Chemical denaturation of holo-proteins. **A:** Urea-induced denaturation of Tr1C (Δ), Tr2C ($\square$), and intact CaM ($\circ$) at $[Ca^{2+}] = 0.3$ mM. **B:** GuHCl-induced denaturation at $[Ca^{2+}] = 1$ mM (symbols as in **A**). **C:** Urea-induced denaturation at $[Mg^{2+}] = 10$ mM (symbols as in **A**). The dotted line in each panel represents the sum of the curves for Tr1C and Tr2C. Unfolding was monitored at 20 °C using far-UV CD. The buffer was 25 mM Tris, pH 8.0, with 100 mM KCl (Ca^{2+} experiments) or 70 mM KCl (Mg^{2+} experiment).

free calcium concentrations for both denaturants. The results obtained using intact CaM with urea and GuHCl are shown in Figure 4; the results for Tr1C and Tr2C with GuHCl are shown in Figure 5. In Table 6, the observed and estimated ligand interaction free energies for Tr1C and Tr2C are compared. The maximum

Table 5. Urea and GuHCl unfolding of holo-proteins (25 mM Tris, 100 mM KCl, pH 8; $[Ca^{2+}] = 0.3$ mM for urea and 1.0 mM for GuHCl)

Transition	Denaturant	Method ^a	ΔG_{20}° ^b (kcal/mol)	$[D]_{1/2}$ (M)
Tr1C	Urea	CD	5.67	6.43
Tr2C	Urea	CD	5.86	6.66
CaM-1 ^c	Urea	CD	4.72	5.36
CaM-2 ^c	Urea	CD	6.45	7.33
CaM-C ^c	Urea	Flu	6.35	7.21
Tr1C	GuHCl	CD	4.95	2.67
Tr2C	GuHCl	CD	5.72	3.1
Tr2C	GuHCl	Flu	5.98	3.23
CaM-1	GuHCl	CD	3.74	2.02
CaM-2	GuHCl	CD	6.32	3.42
CaM-C	GuHCl	Flu	7.11	3.84
CaM-C	GuHCl	Abs	7.18	3.88

^aCD, Flu, and Abs identify unfolding monitored using far-UV CD (220 nm), Tyr138 fluorescence (320 nm), and Tyr138 absorption (288 nm).

^b ΔG_{20}° values were calculated as $-[D]_{1/2} \times m_{av}$, with $m_{av} = -0.88$ kcal/mol M (urea) and -1.85 kcal/mol M (GuHCl) (see Materials and methods).

^cCaM-1 and CaM-2 indicate the first and second transitions resolved for intact CaM (far-UV CD), assigned to the N- and C-domains, respectively (see text). CaM-C represents unfolding of the C-domain in intact CaM (absorption and fluorescence of Tyr138).

interaction free energies, $\Delta\Delta G_b(\text{calc})$, were estimated using published values for the stoichiometric calcium association constants for the fragments (Bayley et al., 1996) in Equation 9. The stabilization conferred by calcium binding $\Delta\Delta G_b(\text{obs})$ is less than expected from the calculated values, especially in the case of GuHCl. This observation can have two possible explanations and either or both could apply: (1) The presence of the denaturant has an effect on the affinity of the fully-folded protein for calcium. This could derive either directly from competition of the denaturant for the calcium-binding sites or indirectly (in the case of GuHCl) from an ionic strength effect. Lowered calcium affinity would reduce the magnitude of the denominator in Equation 8 and hence reduce the interaction free energy. (2) The assumption of negligible binding of calcium to the unfolded state could also be questioned. Equation 7 shows that such binding would limit the interaction free energy observable at high $[Ca^{2+}]$.

To explore the first possibility, calcium binding to the fragments was measured in the presence of GuHCl at concentrations that induced only very limited unfolding of the apo-protein. Initial experiments performed with 5,5'-dibromo BAPTA ($K_{Ca} = 5.7 \times 10^5$ M⁻¹) in the presence of 0.3 M GuHCl showed that the calcium affinity was reduced to the point where it could no longer be measured using this indicator. Therefore, the low affinity indicator, 5-nitro BAPTA, was used and its calcium-binding constants were calibrated in the standard buffer (0.1 M KCl) and in this buffer plus 0.3 M GuHCl or plus an additional 0.3 M KCl, as a control. The calcium binding constant for 5-nitro BAPTA itself is reduced by a factor of ~ 2 at the higher ionic strength with either KCl or GuHCl (see Table 7). In contrast, while calcium binding to the fragments is significantly weakened by both salts, the effect of GuHCl is evidently much greater, since 0.3 M GuHCl causes a reduction in the product of the binding constants ($K_1 \times K_2$) of two orders of

Fig. 4. Chemical denaturation of intact CaM—effect of varying $[Ca^{2+}]$. **A:** Urea-induced denaturation of CaM at $[Ca^{2+}] = 0$ (a), 0.3 (b), 0.6 (c), and 1 mM (d). **B:** GuHCl-induced denaturation of CaM at $[Ca^{2+}] = 0$ (a), 1 (b), 10 (c), and 100 mM (d). Unfolding was monitored at 20 °C using far-UV CD. The buffer was 25 mM Tris, 100 mM KCl, pH 8.

magnitude and 0.4 M KCl causes a reduction of approximately one order of magnitude. The calcium-binding constants for 5-nitro BAPTA and Tr2C were also measured in the presence of 0.3 M urea. At this urea concentration, the affinity of the indicator for calcium is not significantly reduced and the constants of Tr2C are barely affected. Thus, GuHCl exhibits selective effects on the binding of calcium to Tr1C and Tr2C.

These results suggest that the increased ionic strength alone cannot account for the effects of GuHCl, but some competition is implied. Changes in calcium affinity can, in principle, arise from changes in either association or dissociation rate constants. The kinetics of Ca^{2+} dissociation from the C-domain of intact CaM were therefore measured in the presence of different concentrations of KCl, GuHCl, and urea at 20 °C. The dissociation of the two Ca ions occurs with single exponential kinetics, with rates 7.4 ± 0.5 , 8.0 ± 0.5 , and 10.2 ± 0.5 s $^{-1}$ for the C-domain in zero, 100 and 300 mM KCl, respectively. The rates measured in the presence of 0.1 M and 0.2 M GuHCl were 11.8 ± 0.5 and 12.9 ± 0.5 s $^{-1}$, and the rates obtained in the presence of 0.3 M and 3.4 M urea were 8.4 ± 0.5 and 7.3 ± 0.5 s $^{-1}$. These results indicate that the calcium dissociation rates for the C-domain of CaM do not depend strongly on ionic strength and they are not affected to a great extent by the presence of denaturants. Thus, the changes in the equilib-

Fig. 5. GuHCl-induced denaturation of tryptic fragments—effect of varying $[Ca^{2+}]$. **A:** Tr1C at $[Ca^{2+}] = 0$ (a), 1 (b), 10 (c), and 100 mM (d). **B:** Tr2C at $[Ca^{2+}] = 0$ (a), 1 (b), 10 (c), and 100 mM (d). Unfolding was monitored at 20 °C using far-UV CD. The buffer was 25 mM Tris, 100 mM KCl, pH 8.

rium affinity constants for Ca observed in these different solvent conditions are most likely due to changes in the calcium association rates, resulting from both medium (ionic strength) and competition effects.

Effects of magnesium on the stability of calmodulin

The intracellular concentration of Mg^{2+} is of the order of 1 mM, while that of Ca is ~ 0.1 μ M in the resting state and increases to >1 μ M upon stimulation. Several recent studies have concluded that Mg^{2+} binds to CaM and competes with Ca^{2+} binding. Although the reported binding constants show variations, possibly due to different experimental conditions, they are mostly in the range 10^2 to 10^4 M $^{-1}$ and there is good evidence that Mg^{2+} unlike Ca^{2+} binds preferentially to the N-terminal domain (Tsai et al., 1987; Malmendal et al., 1999).

Far-UV CD has been used to study the urea-induced unfolding of CaM and its isolated domains in the presence of 10 mM Mg^{2+} (Fig. 3C; Table 8). Figure 3C shows that Tr1C is significantly more stable than Tr2C in the presence of Mg^{2+} , and it is reasonable to assume that the same order of domain stability is maintained in the intact protein. Thus, Mg^{2+} binding, unlike Ca^{2+} binding, does not reverse the order of domain stability, suggesting that the N-domain

Table 6. Effects of calcium on the urea and GuHCl unfolding of tryptic fragments of CaM (far-UV CD; 25 mM Tris, 100 mM KCl, pH 8, 20 °C)

Protein	Denaturant	[Calcium] (mM)	ΔG_{20}° ^a (kcal/mol)	$\Delta\Delta G_b(\text{obs})$ ^b (kcal/mol)	$\Delta\Delta G_b(\text{calc})$ ^c (kcal/mol)
Tr1C	Urea	0	2.67		
		0.3	5.67	3.00	3.69
Tr2C	Urea	0	2.09		
		0.3	5.86	3.77	6.01
Tr1C	GuHCl	0	3.77		
		1.0	4.95	1.18	5.08
		10.0	7.69	3.92	7.76
		100.0	10.46	6.69	10.44
Tr2C	GuHCl	0	2.65		
		1.0	5.72	3.07	7.41
		10.0	9.47	6.82	10.09
		100.0	11.85	9.20	12.77

^a ΔG_{20}° values were calculated $-[D]_{1/2} \times m_{av}$, with $m_{av} = -0.88$ kcal/mol M (urea) and -1.85 kcal/mol M (GuHCl) (see Materials and methods).

^b $\Delta\Delta G_b(\text{obs})$ was calculated as $\Delta G_{20}^{\circ}(\text{plus calcium}) - \Delta G_{20}^{\circ}(\text{no calcium})$.

^c $\Delta\Delta G_b(\text{calc})$ was calculated as $-RT \ln\{1/(K_1 K_2 [\text{Ca}^{2+}]^2)\}$ (see Materials and methods) with $K_1 K_2 = 6.1 \times 10^9 \text{ M}^{-2}$ for Tr1C and $K_1 K_2 = 3.35 \times 10^{11} \text{ M}^{-2}$ for Tr2C (Bayley et al., 1996).

of CaM binds Mg^{2+} with higher affinity than the C-domain. Stoichiometric binding constants for Mg^{2+} have been calculated using the ligand interaction free energy values ($\Delta\Delta G_b(\text{obs})$) and Equation 8 (see Materials and methods), with the simplifying assumption that there is no cooperativity between the two binding sites within a domain. The values obtained (Table 8) are consistent with the values of 3,500 and 200 M^{-1} reported for Tr1C and Tr2C, respectively, in the absence of KCl (Tsai et al., 1987).

The curves in Figure 3C also show that, as observed for apo-CaM and for Ca^{2+} -CaM, the sum of the unfolding curves for the isolated fragments differs from the unfolding curve for intact CaM. In the presence of 10 mM free Mg^{2+} , the C-domain in intact CaM is destabilized by ~ 0.3 kcal/mol compared with Tr2C and the N-domain is stabilized by ~ 0.7 kcal/mol compared with Tr1C.

Discussion

A comparison of the results obtained with urea and GuHCl shows that these two denaturants give different ΔG° values for both CaM and its isolated fragments. First, as noted above, small (nondenaturating) concentrations of GuHCl ($<0.3 \text{ M}$) stabilize the apo forms of these proteins because of the ionic nature of this denaturant.

Table 8. Effect of 10 mM MgCl_2 on urea unfolding of CaM and its isolated domains (far-UV CD; 25 mM Tris, pH 8)

Transition	Ionic strength ^a (mM)	ΔG_{20}° ^b (M)	$[\text{Urea}]_{1/2}$ (M)	$\Delta\Delta G_b(\text{obs})$ ^c (kcal/mol)	K_1 ^d (M^{-1})	K_2 ^d (M^{-1})
Tr1C	30	4.86	5.52	2.42	1,400	350
	100	4.65	5.28	1.92	840	210
Tr2C	30	2.92	3.32	1.43	483	121
	100	2.80	3.18	0.89	230	57
CaM-1 ^e	100	2.54	2.89	1.09	310	78
CaM-2 ^e	100	5.33	6.06	1.78	722	181

^a Mg^{2+} concentrations were included in the calculation of the ionic strength.

^b ΔG_{20}° values calculated as $-[D]_{1/2} \times m_{av}$, where $m_{av} = -0.88$ kcal/mol M (see Materials and methods).

^c $\Delta\Delta G_b(\text{obs})$ was calculated as $\Delta G_{20}^{\circ}(\text{plus } \text{Mg}^{2+}) - \Delta G_{20}^{\circ}(\text{apo})$ using $\Delta G_{20}^{\circ}(\text{apo})$ values from Table 4.

^d K_1 and K_2 are the estimated stoichiometric association constants for Mg^{2+} , calculated by assuming that $\Delta\Delta G_b(\text{obs}) = -RT \ln\{1/(1 + K_1[\text{Mg}^{2+}] + K_1 K_2[\text{Mg}^{2+}]^2)\}$ (see Materials and methods) and making the assumption that $K_2 = K_1/4$, i.e., that there is no cooperativity between binding sites.

^eCaM-1 and CaM-2 identify the first and second transitions resolved for intact CaM, assigned to the C- and N-domains, respectively (see text).

Second, GuHCl reduces the affinity of these proteins for calcium, even at relatively low concentrations, and thus reduces the stabilizing effects of calcium binding. Comparison of the effects of KCl and GuHCl shows that the reduced calcium affinity derives only partially from ionic strength effects. GuHCl also appears to compete directly with calcium binding, perhaps through direct interaction with the numerous acidic groups involved in the individual calcium binding sites (cf. Pace et al., 1990). Kinetic studies establish that the main effect of GuHCl is on the association rate constants rather than on the dissociation rate constants, consistent with this idea. Therefore, the effects of GuHCl on CaM stability are much more complex than those of urea. We conclude that urea is the preferred denaturant for use with CaM (and with other Ca^{2+} -binding proteins) in quantitating folding/unfolding parameters.

Chemical and thermal denaturation studies on CaM and its isolated domains in the absence of calcium show that these proteins have relatively low stability ($\Delta G_{20}^{\circ} \sim 2\text{--}3$ kcal/mol), which is a common feature with small (noncross-linked) globular proteins. Calculations based on the ΔG° values measured here show that at 37 °C, physiological salt concentrations and pH 8.0 the C-domain of apo-CaM is $\sim 24\%$ unfolded, while the more stable N-domain is less than 2% unfolded.

Knowledge of the conformation of apo-CaM in solution has come from NMR studies (Finn et al., 1995; Kuboniwa et al., 1995; Urbauer et al., 1995; Zhang et al., 1995). These indicate that apo-CaM is a dynamic structure under the conditions used (generally 100 mM KCl, pH 6.5, 25 °C). The present results indicate that the individual domains of apo-CaM are each engaged in a reversible two-state unfolding process, and that the amount of folded apo-N-domain or apo-C-domain will depend on solution conditions, especially ionic strength and temperature. Under conditions of low ionic strength (5 mM KCl), NOE's characteristic of the compact folded structure of the C-domain were lost, and the integrity of the β -structure and of the hydrophobic core was diminished, although

Table 7. Calcium binding constants for 5-nitro BAPTA and the tryptic fragments of CaM (25 mM Tris, pH 8, 20 °C)

Conditions	K_{Ca} (M^{-1}) for 5-nitroBAPTA	$K_1 K_2$ (M^{-2}) for Tr1C	$K_1 K_2$ (M^{-2}) for Tr2C
0.1 M KCl ^a	3.3×10^4	1.45×10^{10}	2.40×10^{11}
0.4 M KCl	1.5×10^4	1.35×10^9	2.65×10^{10}
0.1 M KCl + 0.3 M GuHCl	1.8×10^4	1.55×10^8	2.80×10^9
0.1 M KCl + 0.3 M Urea	3.1×10^4	n.d.	1.85×10^{11}

^aThe constants determined for Tr1C and Tr2C in 0.1 M KCl agree well with those determined using 5,5'-dibromo BAPTA (Bayley et al., 1996).

secondary helical structure was still present (Urbauer et al., 1995). These results are consistent with partial unfolding of the C-domain, as indicated by the observed low degree of stability of this domain to urea and thermal unfolding. Apo-CaM is also known to be significantly (and differentially) more sensitive to proteolytic attack than Ca-CaM (Walsh et al., 1977; Kawasaki et al., 1986; Mackall & Klee, 1991). Unless mitigated in some way, the instability of apo-CaM could therefore enhance its susceptibility to proteolytic cleavage in vivo. In fact, apo-CaM has been reported to interact (more strongly than Ca-CaM) with certain target proteins, notably neuromodulin (Alexander et al., 1987) and neurogranin (Baudier et al., 1991). Such interactions, and possibly those with Mg^{2+} , could provide some protection against this intrinsic instability of the apo-form.

The binding of calcium ion increases the stability of the proteins as predicted by the theory of ligand binding (Schellman, 1975). This stabilizing effect depends on the free calcium concentration as well as on the calcium affinities of both the native and denatured states (see Equation 7). We show here that the C-domain of CaM is the more stable domain for $[Ca^{2+}] = 0.3$ mM, even though the apo-C-domain is thermodynamically less stable than the apo-N-domain (see Fig. 6). This is because the C-domain has a significantly greater affinity for calcium ($K_1K_2 = 6.1 \times 10^9$ M $^{-2}$ for Tr1C and $K_1K_2 = 3.35 \times 10^{11}$ M $^{-2}$ for Tr2C (Bayley et al., 1996)). CaM binds Mg^{2+} with much lower affinity than it does Ca^{2+} (Tsai et al., 1987) and high concentrations of magnesium are therefore required to produce major stabilizing effects. Magnesium, unlike calcium, binds preferentially to the N-domain of CaM (Tsai et al., 1987) and this ensures that the N-domain remains the more stable domain at all Mg^{2+} concentrations (see Fig. 6).

The quantitative analysis of the unfolding profiles from chemical and thermal denaturation experiments reported here shows that the thermodynamic stabilities of the isolated domains are different from those of the corresponding domains in intact CaM. In particular, the C-domain is less stable in intact apo-CaM (and in Mg^{2+} -CaM) than when isolated and the N-domain is more stable in intact apo-CaM (and in Mg^{2+} -CaM) than when isolated. In the presence of 0.3 mM Ca^{2+} , the order in which the domains unfold is reversed (see above) and here we find that it is the N-domain that is less stable in intact Ca^{2+} -CaM than when isolated, while the C-domain is more stable in intact Ca^{2+} -CaM than when isolated. Thus, whichever domain unfolds first in intact CaM appears to be destabilized compared to the corresponding isolated fragment while the second appears to be stabilized compared to the isolated fragment. This is observed in apo-CaM and in the presence of Ca^{2+} and Mg^{2+} , and similar effects are observed in either thermal or chemical denaturation.

Several kinetic and equilibrium studies have shown that the two domains of CaM appear to behave as autonomous units (Martin et al., 1985, 1996; Martin & Bayley, 1986; Suko et al., 1986; Linse et al., 1991). However, interdomain interactions have been postulated in studies of wild-type CaM or SynCaM (Tsalkova & Privalov, 1985; Pedigo & Shea, 1995; Shea et al., 1996; Protasevich et al., 1997) and in studies of CaM in which one domain carries a specific mutation (Martin et al., 1992; Maune et al., 1992b). We do not have direct physical evidence for specific interdomain interactions in the fully folded state of wild-type CaM, but we cannot exclude, a priori, the presence of this kind of interaction. However, the explanation for the observation that the stabilities of the domains in the intact protein differ from those of the isolated fragments does appear to require the general postulate of an unfolding

Fig. 6. Domain unfolding in intact CaM. Schematic representation of the urea-induced unfolding of the N- and C-terminal domains of intact CaM for the (A) apo-protein and for CaM in the presence of (B) 0.3 mM Ca^{2+} or 10 mM Mg^{2+} (C). The curves were calculated using the data of Tables 1, 5, and 8.

intermediate in which there is an interaction between the two domains of a given CaM molecule, one in the folded and one in the unfolded state.

These interactions could be either enthalpic or entropic in origin. Enthalpic interactions might imply a degree of structural specificity, for example, in an interaction between a folded Ca^{2+} -C-domain and an unfolded apo-N-domain. However, the results with apo-CaM and Mg^{2+} -CaM suggest that the reverse situation can also occur; namely, an interaction between a folded Mg^{2+} -N-domain (or a folded apo-N-domain) and an unfolded apo-C-domain. Circular dichroism (this work; Martin & Bayley, 1986) and NMR measurements (Ohki et al., 1997; Malmendal et al., 1999) show that magnesium causes smaller conformational changes in the N-domain than does calcium. In addition, magnesium bind-

ing to either domain does not show hydrophobic exposure by NMR (Ohki et al., 1997), nor by TNS binding (data not shown). Thus, the Mg^{2+} -N-domain (and the apo-N-domain) evidently lack the conformational attributes to form hydrophobic target interactions and, hence, appear intrinsically unlikely to interact specifically with an unfolded apo-C-domain; nevertheless, the effects on domain stability are still observed. A less specific and more general mechanism could reflect the inability of an unfolded apo-domain to attain the same degree of randomness (reflected as the lower entropy of the unfolded chain) when connected to a folded domain in the intact protein. The total free energy (ΔG°) for unfolding the intact protein is close to the sum of the free energies of the isolated domains (Table 2). Thus, the implied stabilization of the intermediate (relative to fully unfolded CaM) would account for decreased ΔG° of the first transition and the increased ΔG° of the second transition of the bimodal unfolding of either apo-CaM or CaM in the presence of Ca^{2+} or Mg^{2+} , compared with ΔG° for the corresponding isolated domains.

The results reported here indicate the importance of separating the contributions of three different factors in the observed stability of a multidomain protein. These are: (1) the intrinsic thermodynamic stability of the individual domains; (2) the effect of differential affinities of individual domains for a given ligand, and the related possibility that a different ligand (e.g., Mg^{2+} as opposed to Ca^{2+}) will show a different pattern of ligand affinities; and (3) the existence of interdomain effects either in the folded or unfolded states of individual domains. When the intrinsic thermodynamic stability is low, which is likely for small nondisulfide cross-linked proteins, then both ligand binding and secondary interactions can have a marked effect on observed stabilities. Thus, one would predict that a small domain sequence excised from a larger protein would have a significantly different stability in the isolated state compared to the same domain sequence in the same protein subjected to a relatively small number of additional interactions with neighboring structures. This suggests that caution is required in dissecting a large protein into smaller components for stability studies, unless they are effectively physically independent in the whole structure. Even in the present case where the domains are largely independent, small but significant interdomain interactions can occur in the unfolding process.

Proteins with low thermodynamic stability may also show increased susceptibility to changes in stability (both positive and negative) induced by mutation. For example, point mutations of a single highly conserved hydrophobic residue in the short β -structure region of any of the four Ca^{2+} -binding loops of CaM cause striking decreases in the T_m (ranging from $\Delta T_m = 50$ to $>80^\circ\text{C}$) for thermal unfolding of the mutated domain, both in the presence and absence of Ca^{2+} (Browne et al., 1997). In extension of the present work, unfolding studies also support the proposal of a (folded/unfolded) intermediate for these mutant proteins (Masino et al., 2000). We conclude that small, noncross-linked protein domains may be attractive candidates for the rational manipulation of protein stability by site directed mutagenesis, a key strategy of protein engineering and design.

Materials and methods

Materials

Drosophila melanogaster CaM expressed in *Escherichia coli* was purified as previously described (Maune et al., 1992b; Browne

et al., 1997). The tryptic fragments of CaM were prepared as described in Barth et al. (1998). HPLC and mass spectrometry confirmed that the N-domain fragment (Tr1C) consisted predominantly ($>95\%$) of residues 1–75 of CaM, and the C-domain fragment (Tr2C) predominantly ($>95\%$) of residues 78–148 (Barth et al., 1998). Concentrations were determined using $\epsilon_{279} = 1,578 \text{ M}^{-1} \text{ cm}^{-1}$ for CaM and Tr2C in the presence of excess calcium (Maune et al., 1992b) and $\epsilon_{259} = 975 \text{ M}^{-1} \text{ cm}^{-1}$ for Tr1C (Barth et al., 1998). Aristar guanidine hydrochloride (GuHCl) and urea were from BDH.

Spectroscopic measurements

Absorption measurements were made using a Cary 3E UV/Visible spectrophotometer. Uncorrected fluorescence emission spectra of the single tyrosine-138 of CaM and Tr2C were recorded using a SPEX FluoroMax fluorimeter with excitation at 280 nm (bandwidth of 1.7 nm) and emission scanned from 290 to 400 nm (bandwidth of 5 nm). Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter as described elsewhere (Bayley et al., 1996). CD intensities are presented as the CD absorption coefficient calculated using the molar concentration of protein ($= \Delta\epsilon_M$) rather than on a mean residue weight (MRW) basis. Values of $\Delta\epsilon_{\text{MRW}}$ may be calculated as $\Delta\epsilon_{\text{MRW}} = \Delta\epsilon_M/N$, where N is the appropriate number of peptide bonds ($= 74, 70$, and 147 for Tr1C, Tr2C, and CaM, respectively).

Calcium binding studies

Stoichiometric calcium association constants for CaM and its tryptic fragments were determined from calcium titrations of apo-protein samples performed in the presence of the chromophoric calcium indicators, 5,5'-dibromo BAPTA and 5-nitro BAPTA, using published methods (Linse et al., 1991; Martin et al., 1996). Measurements were performed at 20°C in 25 mM Tris (pH 8) in the presence of different concentrations of KCl or GuHCl. The calcium binding constant of 5,5'-dibromo BAPTA in the presence of 100 mM KCl is $5.7 \times 10^5 \text{ M}^{-1}$ (Martin et al., 1996). Under the same conditions, the binding constant for 5-nitro BAPTA was found to be $3.3 (\pm 0.4) \times 10^4 \text{ M}^{-1}$ (cf. Pethig et al., 1989).

Denaturation experiments

Chemical denaturation is more than 95% reversible for all the protein samples studied here, since spectroscopic signals characteristic of the native protein are recovered upon dilution of the denaturant to nondenaturing concentrations. Titrations were performed at 20°C in 25 mM Tris, pH 8, 100 mM KCl by adding aliquots of protein in concentrated denaturant to a solution of the protein at the same concentration in aqueous buffer. CD and absorbance signals were measured at 220 and 288 nm, respectively; fluorescence was measured at 320 nm, with excitation at 280 nm. Thermal denaturation was studied by measuring fluorescence at 320 nm in 10 mM HEPES, pH 7.5, 100 mM KCl and by measuring far-UV CD (220 nm) in 25 mM Tris, pH 8.0, 100 mM KCl. Apo protein solutions were heated from 10 to 80°C at $\sim 1^\circ\text{C}/\text{min}$ and the sample temperature was measured with an immersed thermocouple. Reversibility of thermal denaturation was assessed to be $>95\%$ following cooling to 10°C at a rate of $1^\circ\text{C}/\text{min}$.

Data analysis

Chemical denaturation data for the isolated domains (and for intact CaM with absorption and fluorescence measurements of Tyr138) were fitted to a two-state model describing the unfolding as a single transition between native (*N*) and denatured (*D*) states:

$$Y = (I_N + S_N[D])f_N + (I_D + S_D[D])f_D \quad (1)$$

where $I_N(I_D)$ and $S_N(S_D)$ are the intercepts and slopes for the optical signals of the native (denatured) forms, $[D]$ is the denaturant concentration, and $f_N(f_D)$ are the fractions of the total population in the native (denatured) forms. The fractional populations were determined from the equilibrium constant for unfolding, K_u ($= \exp(-\Delta G_u/RT)$), with ΔG_u calculated using the linear extrapolation model (LEM) (Greene & Pace, 1974):

$$\Delta G_u = \Delta G^\circ + \mathbf{m}[D] \quad (2)$$

where ΔG° is the standard free energy of unfolding in the absence of denaturant and $\mathbf{m}$ is the slope that characterizes the change in ΔG_u with $[D]$. The denaturant concentration at the midpoint of the transition $[D]_{1/2}$ is equal $-\Delta G^\circ/\mathbf{m}$. The unknown parameters were obtained from nonlinear least-squares fits directly to the experimental data. It was invariably found that the $[D]_{1/2}$ values were determined more reproducibly than individual ΔG° and $\mathbf{m}$ values, which are highly correlated in the analysis. From more than 25 determinations with Tr1C and Tr2C we calculated average $\mathbf{m}$ values ($\mathbf{m}_{av}$) of -0.88 ± 0.08 kcal/mol M for urea and -1.85 ± 0.13 kcal/mol M for GuHCl. These values are similar to those found for other globular proteins of this size (Myers et al., 1995), and we report individual ΔG° for Tr1C, Tr2C, and the domains in intact CaM calculated from the $[D]_{1/2}$ and $\mathbf{m}_{av}$ values. We estimate that errors in individual ΔG values (not quoted) are of the order of 5–10%.

Thermal denaturation data for the isolated domains (and absorption and fluorescence data with intact CaM) were fitted to the following two-state model:

$$Y = (I_N + S_N T)f_N + (I_D + S_D T)f_D \quad (3)$$

where T is the temperature ($^\circ\text{C}$) and the other parameters are defined as above. Free energy values used in calculating the fractional populations were obtained from the modified Gibbs–Helmholtz equation (Becktel & Schellman, 1987):

$$\Delta G_T^\circ = \Delta H_m(1 - T/T_m) - \Delta C_p(T_m - T + T \ln(T/T_m)) \quad (4)$$

where T_m and ΔH_m are the temperature of the transition midpoint (K) and the van't Hoff enthalpy at T_m , respectively, and ΔC_p is the difference in heat capacity between the native and denatured states.

For denaturation of the intact protein monitored by far-UV CD, the situation is more complex as the signal derives from two domains and we consider the following scheme:

In this scheme, all four ΔG values can, in principle, be different but $\Delta G_1 + \Delta G_3 = \Delta G_2 + \Delta G_4$. Analysis for four ΔG values, however, is not possible. The experimental curves for both chemical and thermal denaturation were therefore analyzed for two ΔG values using a model with a pair of two-state transitions (one for each domain; cf. Protasevich et al., 1997) and using a three-state model with an obligatory intermediate ($N \rightarrow I \rightarrow D$) (cf. Sorensen & Shea, 1998). In all cases where comparisons were made, these two analysis procedures returned essentially identical ΔG values. If, for example, ΔG_2 is substantially greater than ΔG_1 , the two measured ΔG values will be ΔG_1 and ΔG_3 .

Ligand interaction free energy

Ligand binding to the native and/or denatured states of a protein affects the stability of the protein. The change in stability resulting from binding is the ligand interaction free energy $\Delta G_{b(X)}$ given by (Schellman, 1975)

$$\Delta G_{b(X)} = -RT \ln \Sigma_X \quad (5)$$

where Σ_X is the binding polynomial for the ligand to state *X*. For two-state unfolding, the net interaction free energy is the difference in interaction free energies for the native and denatured states:

$$\Delta \Delta G_b = \Delta G_{b(D)} - \Delta G_{b(N)} = -RT \ln(\Sigma_D/\Sigma_N). \quad (6)$$

In the case of calcium binding to an isolated CaM domain, this is given by

$$\Delta \Delta G_b = -RT \ln \left(\frac{1 + K'_1[\text{Ca}] + K'_1 K'_2[\text{Ca}]^2}{1 + K_1[\text{Ca}] + K_1 K_2[\text{Ca}]^2} \right), \quad (7)$$

where $[\text{Ca}]$ is the free calcium concentration and the K_i and K'_i are the stoichiometric calcium binding constants for binding to the native and denatured states of that domain, respectively. Since calcium binds preferentially to the native state, the ligand interaction free energy is stabilizing. The greatest stabilizing free energy is obtained when there is no binding to the denatured state:

$$\Delta \Delta G_b = -RT \ln \left(\frac{1}{1 + K_1[\text{Ca}] + K_1 K_2[\text{Ca}]^2} \right). \quad (8)$$

Since the isolated domains show positive cooperativity in calcium binding (Linse et al., 1991; Martin et al., 1996), K_2 is greater than K_1 and at high calcium concentrations (e.g., $>200 \mu\text{M}$) Equation 8 reduces to

$$\Delta \Delta G_b = -RT \ln \left(\frac{1}{K_1 K_2[\text{Ca}]^2} \right). \quad (9)$$

If calcium does also bind to the unfolded state, then the stabilizing free energy due to calcium binding attains a limiting value at sufficiently high $[\text{Ca}]$ (i.e., at $[\text{Ca}]^2 > 1/(K'_1 K'_2)$), given by

$$\Delta \Delta G_b(\text{limit}) = -RT \ln \left(\frac{K'_1 K'_2}{K_1 K_2} \right). \quad (10)$$

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