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The unusually stable coiled-coil domain of COMP exhibits cold and heat denaturation in 4–6 M guanidinium chloride

Yuan Guo, Richard A. Kammerer, Jürgen Engel*

Abteilung für Biophysikalische Chemie, Biozentrum, Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Abstract

A high thermal stability is observed for the five-stranded α-helical coiled-coil domain of cartilage oligomeric matrix protein COMP. It does not unfold in non-denaturing buffer between 0 and 100° C and thermal denaturation is only achieved at high concentrations of guanidinium chloride (4–6 M). In these solutions the protein structure is lost at decreasing (cold denaturation) and increasing temperatures (heat denaturation). In the cold denaturation region, the melting profile showed deviations from the theory of Privalov et al. [P.L. Privalov, V. Griko Yu, S. Venyaminov, V.P. Kutyshenko, Cold denaturation of myoglobin, J. Mol. Biol. 190 (1986) 487–498] probably due to deviations from a two-state mechanism. High thermal stability as well as cold and heat denaturation was also observed for a mutant of the coiled-coil domain of COMP in which glutamine 54 was replaced by isoleucine but it still forms pentamer. The melting temperatures in plain buffer for the heat denaturation of COMP coiled-coil domain and its mutant obtained by extrapolation to zero molar guanidinium chloride concentration are approximately 160 and 220° C, respectively, which groups them among the most stable proteins.

Keywords: α-Helical coiled coil; Guanidinium chloride; Cold and heat denaturation; Circular dichroism spectroscopy, thermodynamics

1. Introduction

Coiled-coil domains are abundant structures in cytosolic and extracellular proteins and serve the function to connect identical or different subunits of biological macromolecules [5,6,15,17,22,28]. Generally coiled-coil domains are two-stranded in cytosolic proteins and in extracellular matrix proteins three or five right-handed α-helices are intertwisted into a left-handed superhelix. The amino acid sequences of coiled-coil forming peptides share a characteristic heptad repeat $(a-g)_n$ in which positions *a* and *d* are occupied by mostly hydrophobic residues [24,32]. Their side chains participate in hydrophobic contacts between the helices whereas residues in positions *b, c, e, f* and *g* are usually hydrophilic and form the solvent

* Corresponding author. Tel.: +41-61-267-2250; fax: +41-61-267-2189.

E-mail address: juergen.engel@unibas.ch (J. Engel).

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exposed part [22]. It is generally accepted that hydrophobic interactions provide the major stabilizing energy but hydrogen bonds within the α-helices and electrostatic interactions also contribute to coiled-coil stability [1,4,10,14,19,25].

The coiled-coil domain of a protein from the extracellular matrix, cartilage oligomeric matrix protein COMP [26], does not unfold even at the boiling point of water [7,8]. The domain designated COMPcc was prepared by recombinant techniques and its structure was elucidated by X-ray crystallography [23]. COMPcc with seven heptad repeats per chain forms a five-stranded domain with a prominent hydrophobic channel to which hydrophobic molecules including vitamin A and D_3 can bind [12]. The exception of a polar residue in *d* position, glutamine 54, form hydrogen bonds among five subunits and interrupt the hydrophobicity [23]. The α-helices are interlinked by disulfide bonds, which are located at the C-terminus of the domain.

In the present work the stability of COMPcc and its mutant defined as COMPccQ54I in which glutamine 54 was replaced by isoleucine were explored by thermal denaturation in the presence of variable concentrations of guanidinium chloride (GdmCl). In the presence of the denaturant unfolding was observed at increasing and also at decreasing temperatures (cold denaturation). Two denaturation profiles flanked a temperature, $T_{max,}$ at which the maximal ellipticity was observed. This theoretically predicted [2,3,31] phenomenon was observed for a large number of globular proteins [11,30], for collagen-like [9] model peptides [16,20] and data also exist for some designed coiled-coil domains [13,21]. The transitions of COMPcc was only in part fitted by a model based on a difference between specific heat of the native and denatured form [31]. Deviations at low temperature are explained by the presence of an additional conformational state in this region.

2. Experiments

2.1. Protein expression and characterization

COMPcc and COMPccQ54I were prepared and characterized as described by Guo et al. [12]. Proteins were in 20 mM sodium phosphate, 200 mM sodium chloride at pH 7.5.

2.2. GdmCl-induced unfolding and refolding

GdmCl (AA-Grade) was obtained from NIGU (Germany). Stock solution was prepared by dissolving GdmCl to the final concentrations of 20 mM sodium phosphate, 200 mM sodium chloride. The pH was adjusted to 7.5 by small amount of sodium hydroxide. The final concentration of GdmCl was determined from its refractive index according to Pace [27].

COMPcc was incubated with different concentrations of GdmCl. CD signals were recorded at 221 nm at 25° C with a Cary 61 spectropolarimeter (Varian) using a thermostated quartz cell of 1 mm path length. For renaturation, the completely denatured protein in 6.5 M GdmCl was stepwise diluted with buffer to lower the GdmCl concentration. In the experiments, the incubation time was long enough to achieve the equilibrium.

2.3. Thermal unfolding in the presence of GdmCl

Thermal unfolding profiles of COMPcc and COMPccQ54I were monitored by the change of ellipticities at 221 nm as a function of temperature at different concentrations of GdmCl. The experiments were performed as described above for the GdmCl titration.

2.4. Thermodynamic analysis

Thermodynamic analysis were performed according to Privalov [30] by following equations which assume a two-state model of folding from an unfolded state to the completely folded five-stranded coiled-coil structure. The changes of standard values of Gibbs free energy, enthalpy, entropy and specific heat at constant pressure are: ΔG_f°; ΔH_f°; ΔS_f°; and $\Delta c_{p.}$ If the temperature deviates from the standard value of 298.2 K, it is indicated by a subscript at ΔG_f°, ΔH_f°, ΔS_f°. T_m is the midpoint temperature of the transition. Assuming that Δc_p is constant over the temperature interval of integration then:

$$\Delta G_{\mathrm{f}}^{\circ} = \left(1 - \frac{T}{T_m}\right)\Delta H_{\mathrm{f},T_{\mathrm{m}}}^{\circ} + (T - T_{\mathrm{m}})\Delta c_p - T\Delta c_p \ln\frac{T}{T_{\mathrm{m}}} \quad (1)$$

as derived from:

$$\Delta G_{\mathrm{f}}^{\circ} = \Delta H_{\mathrm{f}}^{\circ} - T\Delta S_{\mathrm{f}}^{\circ},$$

with

$$\Delta H_{\mathrm{f}}^{\circ} = \Delta H_{\mathrm{f},T_{\mathrm{m}}}^{\circ} + \int_{T_{\mathrm{m}}}^{T'} \Delta c_p \mathrm{d}T$$

$$\Delta S_{\mathrm{f}}^{\circ} = \Delta S_{\mathrm{f},T_{\mathrm{m}}}^{\circ} + \int_{T_{\mathrm{m}}}^{T'} \frac{\Delta c_p}{T} \mathrm{d}T,$$

and

$$\Delta S_{\mathrm{f},T_{\mathrm{m}}}^{\circ} = \frac{\Delta H_{\mathrm{f},T_{\mathrm{m}}}^{\circ}}{T_{\mathrm{m}}}$$

The fraction of native structure is given by

$$F = \frac{K}{1+K} \quad \text{with} \quad K = e^{-\Delta G_f^{\circ}/RT} \quad (2)$$

where K is the equilibrium constant. The ellipticity signal follows from

$$[\Theta] = F[[\Theta]_{\mathrm{N}} - [\Theta]_{\mathrm{D}} + (A - B)\Delta\vartheta] + [\Theta]_{\mathrm{D}} + B\Delta\vartheta \quad (3)$$

in which $[\Theta]_{\mathrm{N}}$ and $[\Theta]_{\mathrm{D}}$ are the mean molar ellipticities of the native and denatured protein at 20° C, A and B are the slopes of their temperature dependencies and $\Delta\vartheta$ is the difference between the actual temperature and 20° C. In accordance with the experimental observation at low and high GdmCl concentrations, the ellipticity of the denatured state, $[\Theta]_{\mathrm{D}} = -300$ deg cm^2/dmol at 20° C, $A = 26$ and $B = -43$ deg cm^2/dmol, ° C were assumed to be independent on GdmCl concentration. $[\Theta]_{\mathrm{N}}$ was used as an independent parameter and changed linearly from $-25\,000$ deg cm^2/dmol at 1 M GdmCl to $-15\,750$ deg cm^2/dmol at 5 M GdmCl (shown in Table 1).

The Marquardt–Levenberg algorithm [29] of Sigma plot (Jandel Scientific) was employed for curve fitting. T_{m} values of heat denaturation were either evaluated from the best fit or in good agreement directly from the midpoint of the transition. The ellipticity at the midpoint was calculated from the values of $[\Theta]_{\mathrm{N}}$ and $[\Theta]_{\mathrm{D}}$ listed for different GdmCl concentrations in Table 1 and collected for the linear cooperative dependencies by parameters A and B. For COMPccQ54I, T_{m} was only evaluated by the second method. Identical values of $[\Theta]_{\mathrm{N}}$, $[\Theta]_{\mathrm{D}}$, A and B were used for COMPcc and COMPccQ54I in view of the minor difference in composition and structure.

3. Results

Fig. 1 shows the dependence of ellipticity at 221 nm ($[\Theta]_{221}$) on GdmCl concentration at 25° C. To denature 50% of the protein, 3.6 M GdmCl is needed. The unfolding and refolding profiles were identical which demonstrates a complete re-

Table 1
Thermodynamic parameters used for analysis of the thermal transition profiles of COMPcc and COMPccQ54I in the presence of GdmCl

GdmCl concentration (M)	COMPcc				COMPccQ54I		
	0	4	4.5	5	0	5	6.8
$[\Theta]_{\mathrm{N}}$ 20° C (deg cm^2/dmol)		−18250	−17000	−15750			−11700
$[\Theta]_{\mathrm{D}}$ 20° C (deg cm^2/dmol)		−300	−300	−300			−300
$\Delta H_{\mathrm{f},T_{\mathrm{m}}}^{\circ}$ (kJ/mol)		−143	−98	−68			n.d.[a]
Δc_p (kJ/mol K)		−4.0	−4.0	−4.0			n.d.[a]
T_{m} (° C)	(160)[b]	77	67	55	(220)[b]	120[b]	83

[a] n.d.: not determined.
[b] By linear extrapolation with a slope of $\Delta T_{\mathrm{m}}/\Delta C_{\mathrm{GdmCl}} = -22$° C/M.

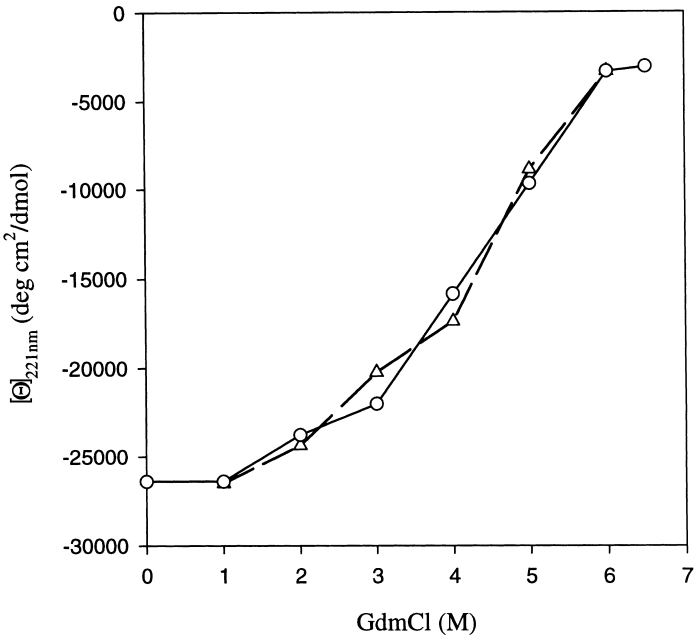

Fig. 1. Denaturation and renaturation curves of COMPcc in GdmCl. For denaturation (circles), the protein was incubated for long enough times at given GdmCl concentrations to reach the equilibrium. For renaturation (triangles), the completely denatured protein in 6.5 M GdmCl was diluted with PBS (pH 7.5) to give GdmCl concentration and equilibrium readings presented in the curve.

versibility. The quite broad transition region suggests a complex denaturation process in which intermediates most likely exist.

Fig. 2a shows the change of $[\Theta]_{221}$ as a function of increasing temperature with different concentrations of GdmCl. The mean molar residue ellipticities at 221 nm increase linearly with increasing temperature and GdmCl concentration before the thermal transition is monitored by a sigmoidal increase of ellipticity. A sigmoidal heat transition with $T_m = 77°$ C is seen at 4 M GdmCl (Fig. 2a). At 4.5 and 5 M GdmCl, the midpoint temperatures of heat denaturation are 67 and 55° C, respectively. At 6 M GdmCl, no transition is visible for COMPcc. This dependence demonstrates that the ellipticity of unfolded protein was also slightly dependent on temperature in a linear way. In addition, clear cold denaturation steps are seen at 4, 4.5 and 5 M GdmCl in the temperature range of 5–40° C. $T_{max,}$ defined as the temperature of maximum helicity at which the minimal value of ΔG_f° is reached [31] is approximately 40° C. Above and below $T_{max,}$ ΔG_f° increases and the stability of the native state decreases. Assuming the two-state model and a constant difference between the specific heat of the folded and unfolded state of Δc_p (see Section 2), only a partial fit was obtained (smooth curves in Fig. 2a). Experimental data at 4 and 4.5 M GdmCl were fitted well at $T > T_{max}$ but at $T < T_{max}$, theoretical curves predicted lower helicities than experimentally observed. With the same values of Δc_p, a less satisfactory fit was observed for the data at 5 M GdmCl. The parameters evaluated from a best fit to all curves are summarized in Table 1.

COMPccQ54I shows a rather similar transition to COMPcc but at a considerably higher GdmCl concentration (6.8 M instead of 5 M) (Fig. 2b). Only partial heat denaturation was observed but it was technically impossible to increase the temperature and GdmCl concentration.

Heat denaturation temperature (T_m) of COMPcc and COMPccQ54I were evaluated as described in Section 2. A direct comparison of T_m

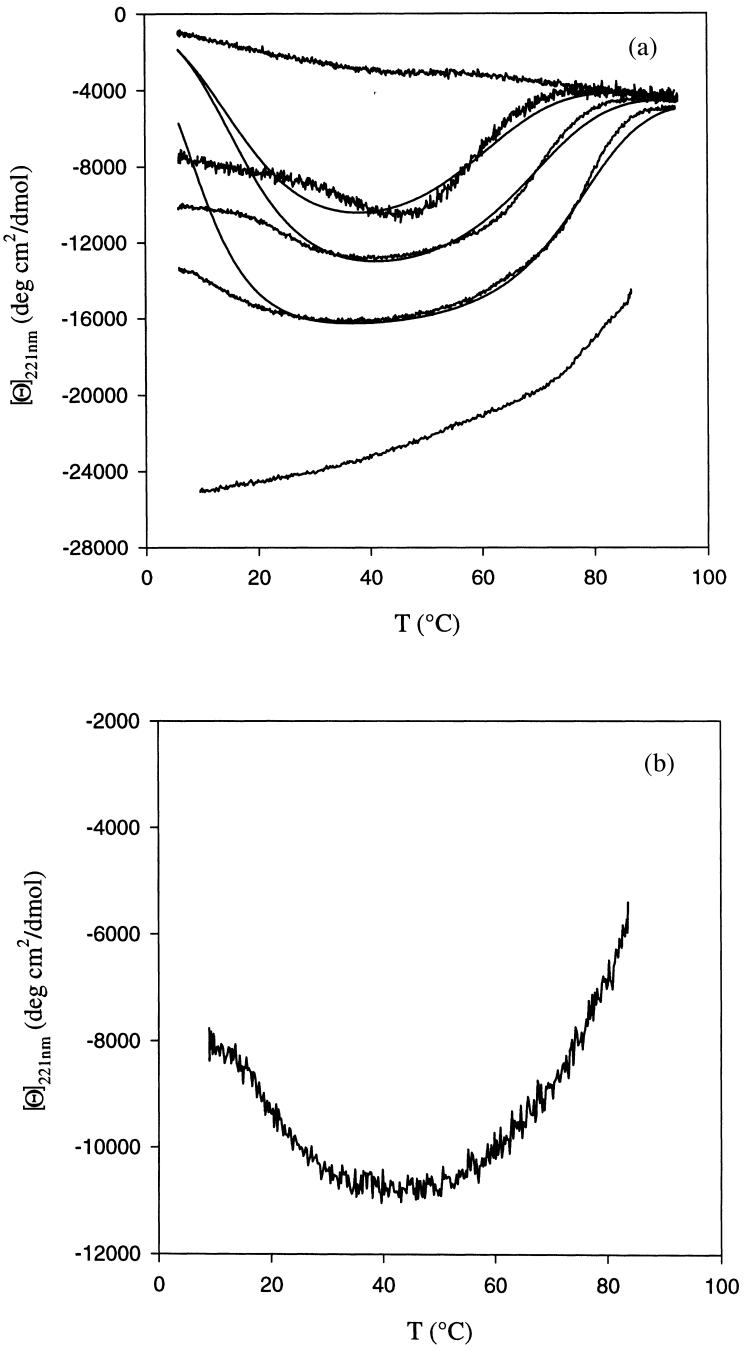

Fig. 2. Thermal denaturation curves of COMPcc (A) and COMPccQ54I (B) in the presence of GdmCl. (a) The noisy curves correspond to GdmCl concentration of 1, 4, 4.5, 5 and 6 M from bottom to top. The smooth curves represent the calculated profiles by assuming a two state transition with $\Delta c_p = -4.0$ (kJ/mol K) and correspond to 4, 4.5 and 5 M GdmCl concentration from bottom to top. (b) Thermal transition of COMPccQ54I in the presence of 6.8 M GdmCl. The parameters used for calculations are listed in Table 1.

values and its mutant is only possible by extrapolation to a common GdmCl concentration. A linear dependence with a slope of $\Delta T_m/\Delta C_{GdmCl} = -22°\,C/M$ was observed for COMPcc (Table 1). Using this value, COMPccQ54I would melt at 120° C in 5 M GdmCl as compared to 55° C for COMPcc at the same concentration of denaturant. The small extrapolation from 6.8 to 5 M GdmCl appears to be justified also in view of linear dependences of T_m on GdmCl concentration observed for other proteins [18]. Experimental dependences are, however, limited to small ranges of GdmCl because of technical restrictions. Large errors may, therefore, be introduced by an extrapolation to zero molar GdmCl using the same construct slope and extrapolated values for zero GdmCl concentration are given in blankets to indicate this uncertainty.

4. Discussion

As demonstrated by Privalov et al. [31], for a two-state model of denaturation, the dependence of ΔH_f° and ΔS_f° on temperature caused by Δc_p leads to a bell-shaped temperature dependence of the free energy, $\Delta G_f^\circ = \Delta H_f^\circ - T\Delta S_f^\circ$. This dependence shows two zero points for ΔG_f° at which folding and unfolding occur with equal probability. Between these values ΔG_f° is negative and the native state is favored. The quantitative dependence of ΔG_f° on absolute temperature T is shown in Eq. (1).

Our experimental data clearly demonstrate a cold and heat denaturation for COMPcc at GdmCl concentrations higher than 4 M. At lower concentrations of the denaturant no transition was observed due to the extreme stability of the coiled-coil domain. Cold denaturations have previously been observed for many globular proteins [11,30] and several synthetic coiled-coil structures [13,21]. For COMPcc the simplest theory assuming only two conformational states and an all-or-none transition between them only describes the phenomenon of cold denaturation in part. For the simple model, identical unfolded states should be reached at both sides of the bell-shaped curves. Fig. 2a clearly shows that this is not the case for COMPcc which exhibits large deviations below T_{max}. A likely reason for this observation is a deviation from the two-state model, which is caused by the five-stranded structure of COMPcc. It has to be expected that the five-stranded coiled-coil structure is formed via intermediates in which a smaller number of strands is connected to a coiled-coil structure. These intermediates may be more stable than the completely unfolded species in GdmCl solution at low temperatures. As mentioned, a certain heterogeneity is also reflected by the rather broad isothermal GdmCl-induced transition curve (Fig. 1) and the observation by Efimov et al. [7] of incomplete disulfide linkage in the COMPcc pentamer.

A striking observation of the present study is the high stability of COMPcc against thermal and denaturant-induced unfolding. This underlines the very efficient stabilization of coiled-coil structures with a high chain number. The observation that the mutant COMPccQ54I is even more stable than COMPcc demonstrates the importance of large buried hydrophobic residues in positions *a* or *d* for stabilization. As it was noticed from the crystal structure of COMPcc, glutamine 54 occupies a position *d* and thus interrupts the hydrophobic interaction pattern of the coiled-coil structure. Replacement of such polar residues by suitable non-polar residues like isoleucine also increased the stability of other coiled-coil protein [21]. The present result clearly shows that the binding of a chloride ion in the channel of COMPcc with glutamine 54 [23] is not essential for the stability of the protein.

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