doi:10.1016/j.jmb.2006.05.075 J. Mol. Biol. (2006) 361, 7–10

JMB

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# Macromolecular Crowding Stabilizes the Molten Globule Form of Apomyoglobin with Respect to Both Cold and Heat Unfolding

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At pH 2 apomyoglobin is extensively unfolded. Addition of increasing concentration of salts has been shown to convert the protein into molten globule form(s), which can undergo both heat-induced and cold-induced unfolding. Increasing concentrations of an inert polymer, dextran, lead to increased formation of molten globule and stabilizes the protein with respect to both heat-induced and cold-induced denaturation. The transitions were studied by circular dichroism. Two-state analysis of the data shows that the effects of salt and polymer are additive, and that stabilization by the polymer is independent of temperature, as predicted by excluded volume theory.



*Keywords:* protein stability; molecular crowding; molten globule; excluded volume; cold denaturation

The effect of macromolecular crowding on protein stability has been considered using a simple statistical-thermodynamic model, which predicts that intra-molecular excluded volume will increase the chemical potential of expanded, unfolded conformations of a protein relative to that of compact native or near-native states.[1] Subsequent studies have shown that high concentrations of inert polymers do stabilize native and molten globule forms of proteins against heat, acid and guanidine hydrochloride-induced unfolding.[2,3] A further prediction of the model is that the extent of stabilization should be only modestly dependent on temperature.[1] Consequently, it can be expected that crowding will protect proteins against cold-induced denaturation. Nishii *et al.* showed that at low pH, the salt induced molten globule forms of apomyoglobin undergo both heat and cold-induced unfolding.[4] We have used this protein as a model system to test the excluded volume model by comparing the effect of crowding on the heat and cold-induced unfolding of the molten globule state.

Figure 1 shows the temperature-dependence of the ellipticity of apomyoglobin at pH 2, in the presence of various concentrations of sodium trichloroacetate (NaTCA) and dextran. In agreement with the results reported by Nishii *et al.*,[4] in the absence of additives, the protein showed low ellipticity, which was almost independent of temperature. At 20 °C, increasing concentrations of both salt and dextran produced increased CD intensity, indicating a shift in the equilibrium towards the molten globule state. An increase or decrease of temperature reverses these changes as the protein unfolds in cooperative transitions.

At low pH, apomyoglobin shows complex behavior. Nishii *et al.* showed that the protein is largely unfolded at room temperature.[4] Addition of salts induces formation of one or more molten globule forms, whose properties depend on the salt (Figure 1).[4] As a first approximation, we introduce a simple two-state model of the effect of different co-solutes upon the conformational state of the protein, on the basis of the following assumptions.

(1) At pH 2, the protein is assumed to exist in either of two states, molten globule (M) or unfolded (U). It is understood that these effective states are conformational ensembles characterized by reasonably well-defined thermodynamic properties, rather than microstates representing unique conformations. The equilibrium between these states is described by:

$$K_{MU} \equiv [U]/[M] = f_U/(1 - f_U) = \exp(-\Delta G_{MU}/RT) \quad (1)$$

Abbreviation used: NaTCA, sodium trichloroacetate.

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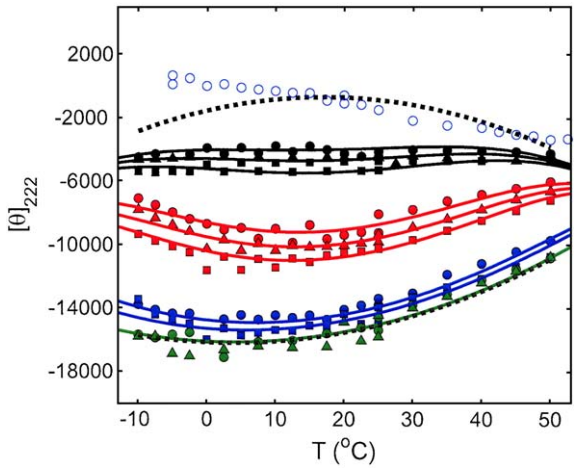


**Figure 1.** Horse heart myoglobin and dextran (average molecular mass 30,000 Da) were obtained from Sigma Chemical Co (St. Louis, MO). Apomyoglobin was prepared by the method of Hapner *et al.*[5] and stored on ice. Circular dichroism measurements were made in a Jasco J-715 spectropolarimeter, equipped with a computer-controlled Neslab RTE-111 circulating waterbath, which maintained the temperature of the cell holder. Heat and cold denaturation transitions were measured separately, using the ellipticity at 222 nm as a measure of helical structure. A 1 mg/ml solution of protein was diluted tenfold into the required buffers (10 mM HCl, plus sodium trichloroacetate (NaTCA), and dextran), in a 1 mm pathlength cuvette at 20 °C. Following measurement of ellipticity for 5 min at each temperature, the temperature of the cuvette was changed by a small amount (2.5–5 °C) and the solution was equilibrated at the new temperature for 15 min before the ellipticity was remeasured. The average value at each temperature was converted into mean residue ellipticity for analysis. At the end of the experiment, the solution was returned to 20 °C. Under all conditions, the observed changes in ellipticity were more than 90% reversible. The temperature-dependence of the mean residue ellipticity at 222 nm of apomyoglobin solutions (all 9 mM HCl): (○) 8 M urea; black symbols, no NaTCA; red symbols, 9 mM NaTCA; blue symbols, 23 mM NaTCA; green symbols 45 mM NaTCA. Dextran concentrations: (●) none; (◆) 135 mg/ml; (■) 270 mg/ml. Continuous curves are calculated from the two-state model using best-fit values of $u_0 = -1551$; $u_1 = 100$; $u_2 = -2.92$; $m_0 = -1623$; $m_1 = -10.8$; $m_2 = 2.41$; $k_0 = -0.693$; $k_1 = 0.0155$; $k_2 = -4.13 \times 10^{-4}$; $\alpha = 0.169$; $\beta = 0.00184$. Broken curves are the predicted behavior of U and M from equations (7) and (8).

where $f_U$ is the mass fraction of unfolded states, $\Delta G_{MU}$ is the standard state free energy change for unfolding of the M state, $R$ is the molar gas constant and $T$ is the absolute temperature.

(2) It is assumed that the free energy of unfolding is a function of temperature and the concentrations of each cosolute. For simplicity, the entropic part of the free energy change is assumed to vary with the concentration of each cosolute in an approximately linear fashion, as postulated by excluded volume theory:

$$\Delta G_{MU}(T, c_{TCA}, w_{dex}) = \Delta G^0_{MU}(T) + RT(\alpha c_{TCA} + \beta w_{dex}) \quad (2)$$

where $c_{TCA}$ denotes the molar concentration of NaTCA and $w_{dex}$ is the concentration (w/v) of dextran. Combination of equations (1) and (2) yields:

$$K_{MU}(T, c_{TCA}, w_{dex}) = K^0_{MU}(T)\exp(-\alpha c_{TCA})\exp(-\beta w_{dex}) \quad (3)$$

where $K^0_{MU}$ denotes the value of $K_{MU}$ in the absence of either NaTCA or dextran. It follows from equations (1) and (3) that the mass fraction of protein in the U state is given by:

$$f_U(T, c_{TCA}, w_{dex}) = \frac{K_{MU}(T, c_{TCA}, w_{dex})}{1 + K_{MU}(T, c_{TCA}, w_{dex})} \quad (4)$$

(3) The ellipticity at 222 nm is assumed to be a mass average of the ellipticities of M and U, which are themselves assumed to be independent of cosolute and vary only with temperature:

$$[\theta]_{222}(T, c_{TCA.}, w_{dex}) = f_u(T, c_{TCA}, w_{dex})[\theta]_{U,222}(T) + [1 - f_u(T, c_{TCA}, w_{dex})][\theta]_{M,222} \quad (5)$$

(4) $K^0_{MU}$ is assumed to vary smoothly with temperature in a manner that may be described empirically by:

$$\log_{10} K^0_{MU}(T^*) = k_0 + k_1 T^* + k_2 T^{*2} \quad (6)$$

where $T^*$ is expressed in degrees Celsius.

(4) $[\theta]_{U,222}$ and $[\theta]_{M,222}$ are assumed to vary smoothly with temperature in a manner that may be described empirically by quadratic functions:[6]

$$[\theta]_{U,222}(T^*) = u_0 + u_1\ T^* + u_2\ T^{*2} \quad (7)$$

$$[\theta]_{M,222}(T^*) = m_0 + m_1\ T^* + m_2\ T^{*2} \quad (8)$$

where $T^*$ is expressed in degrees Celsius.

Equations (3) to (8) permit us to calculate the ellipticity of apomyoglobin at pH 2 as a function of temperature and the concentrations of NaTCA and dextran using 11 undetermined parameters: $\alpha$, $\beta$, $k_0$, $k_1$, $k_2$, $u_0$, $u_1$, $u_2$, $m_o$, $m_1$, and $m_2$. We have globally fit this model to a comprehensive data set consisting of ellipticity measurements carried out between −10 °C and 50 °C at 11 different combinations of concentrations of NaTCA and dextran. Non-linear least-squares modeling yielded best-fit values of the 11 undetermined parameters listed above. The data, together with calculated best-fit curves, are presented in Figure 1; also plotted are the calculated ellipticities of the pure U and M states.

Several interesting conclusions emerge from our data coupled with the two-state model analysis. The first is that under the conditions of these experiments, there appears to be a significant mass fraction (perhaps as large as 15–20%, depending upon temperature) of molten globule state at pH 2 even in the absence of NaTCA or dextran. This interpretation conflicts with the assumption made by Nishii *et al.* that apomyoglobin is completely unfolded at pH 2 in the absence of NaTCA.[4] Therefore, we