Annals of Biomedical Engineering, Vol. 33, No. 8, August 2005 (© 2005) pp. 1125–1131
DOI: 10.1007/s10439-005-5780-8

Effects of Crowding on the Thermal Stability of Heterogeneous Protein Solutions

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(Received 24 September 2004; accepted 15 April 2005)

Abstract—Crowding can substantially affect the transition of a protein between its native (N) and unfolded (U) states via volume exclusion effects. Also, it influences considerably the aggregation (A) of unfolded proteins. To examine the details, we developed an approach for computing the kinetic rates of the process $N \leftrightarrow U \rightarrow A$ in which the concentration of the protein is explicitly taken into account. We then compute the relative change with temperature of the protein denaturation for various fractional volume occupancies and partition of proteins in solution. The analysis indicates that, in protein solutions in which the average distance between proteins is comparable with the radius of gyration of an unfolded protein, steric effects increase the stability of the proteins which are in compact, native states. In heterogeneous protein solutions containing various types of proteins with different thermal stabilities, the unfolding of the most thermolabile proteins will increase the stability of the other proteins. The results shed light on the way proteins change the thermal stability of a cell as they unfold and aggregate. This study may be valuable in questions related to the dynamics of thermal injuries.

Keywords—Thermal injury, Protein denaturation, Lumry–Eyring model, Crowding effects.

INTRODUCTION

The analysis of the heat transfer occurring in burn injuries[8–11,16,17,28] revealed that the characteristic temperature profiles lie in the domain of the thermal instability of vital cellular components.[7] Depending on the time of exposure at supraphysiological temperatures, proteins and cell organelles can undergo structural modifications and irreversible denaturation which may trigger cell death. Despite the effort, the amount of denaturated proteins leading to thermal death as well as critical targets for cell killing are not precisely established yet.[18] Recently,[7] we employed a standard chemical physics approach to estimate the degree of alteration of vital cellular components for characteristic temperature profiles of a 90°C (20 s contact) burn. Our simulations suggest that the thermal alteration of the plasma membrane is likely to be the most significant cause of the tissue necrosis. The *lipid bilayer* and *membrane-bound ATPases* have a high probability of thermal damage.

Our study showed that the vulnerability to thermal denaturation of each cellular component can efficiently be characterized by two main thermodynamic parameters, the melting temperature and denaturation enthalpy.[7] These parameters can be obtained by calorimetric measurements of proteins in dilute solutions.[24] In principle, they also can describe fairly well the *in situ* thermal protein denaturation after applying a correction due to cell crowding effects.[7,26] However, there remain important features of the thermodynamical behavior of proteins in a crowded environment that need further elucidation. One aspect which has to be analyzed is the self-stabilization effect. If the protein interspace becomes comparable with the radius of gyration of an unfolded protein, then the subsequent confinement due to volume excluded by the unfolded protein would provide stability for adjacent proteins which are in compact, native states. This effect can usually be observed in manufacturing artificial skin. Typically, Type I *collagen* has melting temperatures (gelatinization) between 50 and 60°C in various dilute aqueous solutions. If water is removed by lyophylization, temperatures of 105°C can be well tolerated for 24 h without major denaturation of the protein content.[38]

Another aspect is related to the behavior of solutions of mixtures of proteins with different thermal stabilities. An interesting question here is how the unfolding and subsequent aggregation of thermally labile proteins affect the stability of other proteins in the mixture which have higher midpoint transitions (50% denaturation). The phenomena related to the thermal behavior of proteins in crowded solutions have important implications in understanding the behavior of cells and tissues at supraphysiological temperatures. Particularly, they might shed light on the issue of the damage accumulation in thermally injured tissues, which is a long-standing clinical problem.[7–11,16,17] We propose an efficient method to estimate the time–temperature dependence of thermal denaturation of proteins in a crowded

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heterogeneous solution. The model resembles the cell in its multicomponent aspect. The implications of the model are examined here in terms of the dependence of protein denaturation on crowding effects, including high protein concentration and increase of the volume available per protein after unfolding, as well as kinetics of aggregation. Within the present approach one can ascertain separately contributions to the damage accumulation from various proteins having different thermal stabilities and interacting with each other.

METHODS

The kinetic model for the thermal denaturation of the cellular components[7] is revisited in the present paper. We extend considerations of the reversible thermodynamical transitions from the native (N) to unfolded (U) states, N $\leftrightarrow$ U, to the generalized Lumry–Eyring model[21,29] N $\leftrightarrow$ U $\rightarrow$ A, where A represents a final state which results from the irreversible aggregation unfolded (U) proteins. We present a short description of the Lumry–Eyring model in the first subsection of the paper.

Within the present model, we assume that crowding affects protein dynamics via steric effects. Therefore, it plays an important role in setting the kinetic rates of the reversible part (N $\leftrightarrow$ U) of the reaction. In the second subsection of the paper we recall the basic physical chemistry approach for computing kinetic rates in the limit of high dilution. In third subsection we introduce corrections on these kinetic rates due to crowding effects.

The irreversible step U $\rightarrow$ A is regarded as a protein aggregation process which is limited by the diffusion of the unfolded proteins. This picture is based on experimental evidence demonstrating that, when unfolded proteins collide, they can easily form stable aggregates via hydrophobic interactions.[24] A method for deriving the rate of aggregation of unfolded proteins is described in the fourth subsection.

The Lumry–Eyring Model

To monitor the aggregation of denaturated proteins with the temperature, we write the kinetic equations corresponding to the reaction model N $\leftrightarrow$ U $\rightarrow$ A in the form

$$\begin{aligned}\frac{\partial P_{\mathrm{N},i}}{\partial t} &= k_{\mathrm{f},i}P_{\mathrm{U},i} - k_{\mathrm{u},i}P_{\mathrm{N},i}\\ \frac{\partial P_{\mathrm{U},i}}{\partial t} &= -k_{\mathrm{f},i}P_{\mathrm{U},i} + k_{\mathrm{u},i}P_{\mathrm{N},i} - k_{\mathrm{a},i}P_{\mathrm{U},i}.\\ P_{\mathrm{A},i} &= 1 - P_{\mathrm{N},i} - P_{\mathrm{U},i}\end{aligned} \tag{1}$$

$k_{\mathrm{u},i}$ and $k_{\mathrm{f},i}$ are the unfolding/folding rate coefficients, $k_{\mathrm{a},i}$ denotes the rate constant for the irreversible aggregation and $P_{\mathrm{N},i}$, $P_{\mathrm{U},i}$ and $P_{\mathrm{A},i}$ represents the distribution density of the population in the native, unfolded and aggregated state of the protein species i. At initial time, $t = 0$, we assume $P_{\mathrm{N},i} = 1$, $P_{\mathrm{U},i} = 0$ and $P_{\mathrm{A},i} = 0$. This is a model for an experiment in which temperature is changed with time according to a "temperature history" $T(t)$.[7–11,16,17,28] The main assumption in above is that the unfolding step is not at equilibrium. Equations (1) represent the general form of the Lumry–Eyring model for irreversible denaturation.[21] The model is generally used to interpret the departure of the DSC results from those predicted by a reversible two-state approach.[29,37]

The Rates of Folding/Unfolding of Protein in the Limit of High Dilution

A thorough understanding of the protein dynamics requires a detailed description of the thermodynamic driving force. Generally, this is governed by the difference in the Gibbs free energy, ΔG, between the states involved in transition. ΔG includes contributions from the interaction with other proteins in solution which is reflected in the thermodynamic activity coefficient $\gamma_{i,\mathrm{N(U)}}$[26]

$$\ln \gamma_{i,N(U)} = \frac{1}{RT}\left[\left(\frac{\partial G}{\partial w_i}\right)_{\{w\}} - \left(\frac{\partial G}{\partial w_i}\right)_{\{w\}\rightarrow 0}\right], \tag{2}$$

where N(U) refers to the native or unfolded state of the protein specie i and $\{w\}$ denotes the composition of the solution (i.e., the equilibrium concentrations of all solutes).

In the limit of high dilution, γ_i approaches unity. Under such experimental conditions, reversible unfolding/folding transitions of single protein molecules can be studied at equilibrium where the protein spends an equal amount of time on the two sides of the folding barrier [$P_{\mathrm{N}}(t) = P_{\mathrm{U}}(t)$].[3] For a two-state folder characterized by the unfolding/folding rate coefficients k_{u} and k_{f}, this happens at the thermodynamic midtransition (see below) at which the equilibrium constant K_0 is unity. Generally, K_0 characterizes the balance between the enthalpy and entropy changes ΔH_{un} and ΔS_{un} during unfolding[24]

$$K_0 \equiv \frac{k_{\mathrm{u}}}{k_{\mathrm{f}}} = \exp\left(-\frac{\Delta H_{\mathrm{un}}}{RT_m}\right)\exp\left(\frac{\Delta S_{\mathrm{un}}}{R}\right). \tag{3}$$

ΔH_{un} and ΔS_{un} are function of temperature and can be written as[24]

$$\Delta H_{\mathrm{un}}(T) = \Delta H_{\mathrm{un}}(T_m) + \int_{T_m}^{T} dT\,\Delta C_{\mathrm{p}}^{\mathrm{un}}(T), \tag{4}$$

$$\begin{aligned}\Delta S_{\mathrm{un}}(T) &= \frac{\Delta H_{\mathrm{un}}(T_{\mathrm{m}})}{T_{\mathrm{m}}} + \int_{T_{\mathrm{m}}}^{T} d\ln(T)\,\Delta C_{\mathrm{p}}^{\mathrm{un}}(T)\\ &\cong \frac{\Delta H_{\mathrm{un}}(T_{\mathrm{m}})}{T_{\mathrm{m}}} + \Delta C_{\mathrm{p}}^{\mathrm{un}}\ln\left(\frac{T}{T_{\mathrm{m}}}\right),\end{aligned} \tag{5}$$

where $\Delta C_{\mathrm{p}}^{\mathrm{un}}(T)$ is the heat capacity difference between the native state and unfolded state of the protein. The effect of temperature on $\Delta C_{\mathrm{p}}^{\mathrm{un}}(T)$ is small and, generally, the value of $\Delta C_{\mathrm{p}}^{\mathrm{un}}$ for most of protein systems can be approximated

[3] Note that, in the limit of high dilution, $P_{\mathrm{A},i} = 0$ and $k_{\mathrm{a},i} = 0$ in Eqs. (1).

by the gas constant $R \cong 8.315\ \mathrm{J\,K^{-1}\,mol^{-1}}$.[24] The equilibrium constant (3) becomes

$$K_0 = \exp\left(-\frac{\Delta H_{\mathrm{un}}(T_{\mathrm{m}})}{RT}\left(1-\frac{T}{T_{\mathrm{m}}}\right) - \left(1-\frac{T_{\mathrm{m}}}{T}-\frac{T}{T_{\mathrm{m}}}\ln\frac{T}{T_{\mathrm{m}}}\right)\right), \quad (6)$$

which is unity at $T = T_{\mathrm{m}}$. T_{m} is an important parameter for characterizing the thermal stability of the protein. Differential scanning calorimetry (DSC) provides an experimental means to measure T_{m} along with the enthalpy and entropy changes at the midpoint transition.[24]

It was shown[30] that the Gibbs free energy at any temperature in the neighborhood of the unfolding event ($T = T_{\mathrm{m}}$) can be derived in terms of the corresponding function G_{m} evaluated at T_{m}

$$\frac{G}{RT} = \frac{G_{\mathrm{m}}}{RT_{\mathrm{m}}} + \frac{H}{R}\left(\frac{1}{T}-\frac{1}{T_{\mathrm{m}}}\right). \quad (7)$$

The change of the free energy at the transition point ($T = T_{\mathrm{m}}$) is zero, $\Delta G_{\mathrm{m}} = 0$. In this case, the rate coefficient of protein unfolding k_{u} is also a function of T_{m}

$$k_{\mathrm{u}} = \text{const}\ \exp\left(-\frac{\Delta G_{\mathrm{un}}}{RT}\right) = \text{const} \times \exp\left(-\frac{\Delta H_{\mathrm{un}}}{RT}\left(1-\frac{T}{T_{\mathrm{m}}}\right)\right). \quad (8)$$

A is a constant that depends on the coupling of the protein with the solvent.[4] Under such circumstances, the backward rate is simply

$$k_{\mathrm{f}} = \frac{k_{\mathrm{u}}}{K_0}. \quad (9)$$

Experimental papers in the field skip often to report the value for the entropy of unfolding, ΔS_{un}. The above equations show how the equilibrium thermodynamic parameters T_{m}, ΔH_{un} and ΔS_{un} are related to each other and provide an alternative way for expressing the kinetic rates k_{u} and k_{f} as a function of only T_{m} and ΔH_{un}. Based on the values of the kinetic rates k_{u} and k_{f}, one can easily derive the time evolution of probability density distributions in the native (P_{N}) and unfolded (P_{U}) states for various temperatures. The above approach can be extended to include properties of the coupling between the protein and solvent.[3–6]

Highly Volume Occupied Solutions

Effects of Crowding on the Unfolding/Folding Kinetic Rates

Obviously, the above picture is not valid for the cytoplasm of cells. Inside the cell, there are a large number of soluble and insoluble macromolecules at different concentrations, sometimes at very high concentrations depending on physiological and environmental conditions. Physiological fluid media contain macromolecules collectively occupying between a lower limit of about $f = 7\%$ and an upper limit of about $f = 40\%$ of the total fluid volume.[14] In this case, the activity coefficient γ_i of each macrosolute deviates from unity by as much as several orders of magnitude, with a potentially major impact on reaction equilibria and rates.

In the following we describe crowding effects in a homogeneous protein solution.[35] The radius of gyration r_{g} of a protein (the radial size which defines how compact the protein structure is) expands during unfolding excluding volume to other surrounding proteins. If the increase Δr of the radius of gyration is in the range of the protein interspace, defined as the mean distance between proteins in solution [$d = (3/4\,\pi\, n)^{-1/3}$, where n is the protein concentration], the subsequent confinement would provide stability for adjacent proteins which are in compact, native states. The self-stabilization effect develops progressively during protein unfolding. The effect, which is described here for the case of a protein solution, is expected to occur also in tightly packed fibers and membranes.[38] To express this effect in a more quantitative way, we write the apparent equilibrium constant K as a function of the activity coefficients of the molecules in their native and unfolded states, γ_{N} and γ_{U}[26]

$$K = K_0\frac{\gamma_{\mathrm{N}}}{\gamma_{\mathrm{U}}}. \quad (10)$$

(K_0 is defined by Eq. (6).) For proteins in native states interacting exclusively *via* steric repulsion forces the activity coefficient is $\gamma_{\mathrm{N}} = V_{\mathrm{tot}}/V_{\mathrm{av,N}}$, where V_{tot} and $V_{\mathrm{av},N}$ denote the total volume and volume available per native protein. V_{tot} can be written as $V_{\mathrm{tot}} = N_{\mathrm{p}}v_{\mathrm{p}}^{\mathrm{eff}} \cong N_{\mathrm{p}}4\pi/3d^3$, where N_{p} is the number of proteins in solution, each protein occupying an effective volume $v_{\mathrm{p}}^{\mathrm{eff}} \cong 4\pi/3d^3$, dr_{N}. r_{N} stands for the radial size of the molecule in the native form. The available volume $V_{\mathrm{av,N}}$ is the volume in which the center of an additional molecule of that solute may be placed.[26] We can approximate $V_{\mathrm{av,N}}$ by $V_{\mathrm{av,N}} \cong 4\pi/3(d - r_{\mathrm{N}})^3$. The apparent activity for the unfolded state can be defined in a similar manner as $\gamma_{\mathrm{U}} = V_{\mathrm{tot}}/V_{\mathrm{av,U}}$, where $V_{\mathrm{av,U}}$ represents the volume available for a protein in the unfolded state $V_{\mathrm{av,U}} \cong 4\pi/3(d - r_{\mathrm{N}} - \Delta r)^3$. Equation (10) becomes

$$\frac{K}{K_0} = \left(1-\frac{\Delta r}{r_{\mathrm{N}}}\frac{1}{\frac{d}{r_{\mathrm{N}}}-1}\right)^3. \quad (11)$$

Further, we can approximate d/r_{N} by

$$\frac{d}{r_{\mathrm{N}}} \cong \left(\frac{3}{4\pi N_{\mathrm{p}}}\frac{V_{\mathrm{tot}}}{r_{\mathrm{N}}^3}\right)^{1/3} = f^{-1/3}, \quad (12)$$

[4] *const* sets the relative time scale of the unfolding event. The relevant time scale may depend on the case of interest (of the order of minutes for typical DSC experiments and seconds for usual thermal burns).

where f represents the fractional volume occupancy of the protein, and cast Eq. (11) in the form

$$\frac{K}{K_0} = \left(1 + \frac{\Delta r}{r_N}\frac{1}{1 - f^{-1/3}}\right)^3. \quad (13)$$

By looking at Eq. (12), it is not difficult to see that any conformational change that increases the volume of a protein ($\frac{4\pi}{3}r_N^3 \to \frac{4\pi}{3}(r_N + \Delta r)^3$) changes the protein interspace d. Consequently, we must relate d to the evolution of the density distribution of the population in the unfolded state of the protein P_U, $P_U = 1 - P_N$. It follows that

$$\frac{d}{r_N} \cong \left(\frac{V_{tot}}{N_p\left[P_N\frac{4\pi}{3}r_N^3 + (1 - P_N)\frac{4\pi}{3}(r_N + \Delta r)^3\right]}\right)^{1/3} = \left[f P_N + f(1 - P_N)\left(1 + \frac{\Delta r}{r_N}\right)^3\right]^{-1/3}, \quad (14)$$

and Eq. (13) can be written as

$$\frac{K}{K_0} = \left(1 + \frac{\Delta r}{r_N}\frac{1}{1 - \left(f\ P_N + f\ (1 - P_N)\left(1 + \frac{\Delta r}{r_N}\right)\right)^{-1/3}}\right)^3, \quad (15)$$

Equation (15) shows that protein unfolding is progressively inhibited by the conformational change that increases the characteristic volume of the protein.

We can also write a more general form for Eq. (15) which can be used to describe the unfolding of a protein species i in a crowd formed by M various other protein species. This is

$$K_i = K_{0,i}\left(1 + \frac{\Delta r_i}{r_{N,i}}\frac{1}{1 - f\sum_j^M x_j\left[\left(P_{N,j} + (1 - P_{N,j})\left(1 + \frac{\Delta r_j}{r_{N,j}}\right)\right)^{-1/3}\right]}\right)^3, \quad (16)$$

where x_j stands for the partition of various protein species in the solution, $x_j = N_{p,j}/N_p$ (i.e., the number of molecules of species j, $N_{p,j}$, versus the total number of molecules in the solution, N_p, $\sum_j^M n_j = 1$). Eq. (16) shows that the volume exclusion effect leads to a decrease of the equilibrium constant K_i of the protein species i in a $f\sum_j^M n_j$ solution of different protein kinds j.

The Rate of Aggregation of Unfolded Proteins

Little is actually known about the energetics of the irreversible step U → A. Protein aggregation is generally believed to be caused by hydrophobic interactions. Unfolded proteins have their hydrophobic residues exposed to water. They stick together to minimize the area of hydrophobic exposure. Therefore, the tendency of aggregation increases with the increase of the population in the unfolded state. The rate of aggregation is limited by the diffusion of the unfolded proteins and can be approximated by the inverse of the diffusion time τ_i, $k_{a,i} = \frac{1}{\tau_i}$. τ_i for an unfolded molecule of a protein species i relates to its corresponding diffusion coefficient D_i by $\tau_i = \frac{d'^2}{4D_i}$. Here, D_i is an effective diffusion coefficient which includes a correction[23] due to the restriction on the movement of the molecules in a crowded environment

$$D_i = \left(\frac{1 - f}{1 + f}\right)^2 D_{0,i} = \left(\frac{1 - f}{1 + f}\right)^2 \frac{kT}{6\pi\eta_0(r_{N,i} + \Delta r_i)}. \quad (17)$$

$D_{0,i}$ represents the diffusion coefficient in a dilute solution, which is expressed in the right-hand part of Eq. (17) in terms of the radius of the molecule ($r_{N,i} + \Delta r_i$), temperature T and viscosity coefficient η_0 by the Stokes–Einstein relationship. Here, we assume that the aggregation applies only to unfolded proteins and consider that the translational diffusion length d' depends on the concentration of all unfolded proteins. Therefore, we can write

$$d' = \left(\frac{3}{4\pi}\right)^{1/3}\left[\frac{1}{V_{tot}}\sum_j^M N_{p,j}(1 - P_{N,j})\right]^{-1/3} = \left[f\sum_j^M x_j\frac{3}{4\pi r_{N,j}^3}(1 - P_{N,j})\right]^{-1/3}. \quad (18)$$

We now can express quantitatively the rate of the irreversible step $k_{a,i}$ by

$$k_{a,i} \cong \frac{2}{3\pi}\left(\frac{1 - f}{1 + f}\right)^2 \frac{kT\left[f\sum_j^M x_j(1 - P_{N,j})\right]^{2/3}}{\eta_0 r_{N,i}^3\left(1 + \frac{\Delta r_i}{r_{N,i}}\right)}. \quad (19)$$

As we can see, $k_{a,i}$ is not a common quantity for all proteins. Within the present diffusion limited approach, this depends naturally on the size of the protein.

RESULTS AND DISCUSSION

We note that $k_{f,i} = \frac{k_{u,i}}{K_i}$), where K_i is given by Eq. (16), and $k_{a,i}$ (see, Eq. (19)) depend explicitly on the distribution densities of the population in the native state $P_{N,i}$. Equations (1) are non-linear differential equation and have to be solved numerically. In the present model, we assume that the unfolding and the diffusion processes occur on the same time scale, which renders const $\sim 10^7\ s^{-1}$ (the pre-exponential factor of the Arrhenius kinetic rate). The value of viscosity coefficient η_0 in a dilute aqueous solution is roughly equal to $\eta_0 \cong 1$ cP in which a protein with radius of gyration $r_N \cong 0.5$ nm has a diffusion time equal $\tau_1 \cong 0.19$ ns at 27°C. We characterize the behavior of several test protein solutions for conditions which reflect a certain temperature history. As an example, we use here a time–temperature dependence corresponding to a deep muscle electrical shock injury of 10 kV, 1 s hand-to-hand

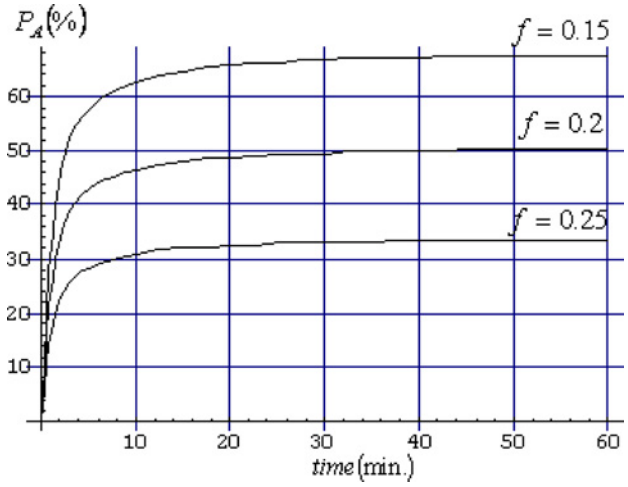

FIGURE 1. Proteins become thermally more stable with increasing the extent of crowding. Denaturation of *adenylate kinase* in time, at various volume occupancies, for a temperature history as reported in Ref.[13] (a muscle electrical shock injury of 10 kV, 1 s hand-to-hand contact at the distal forearm location).

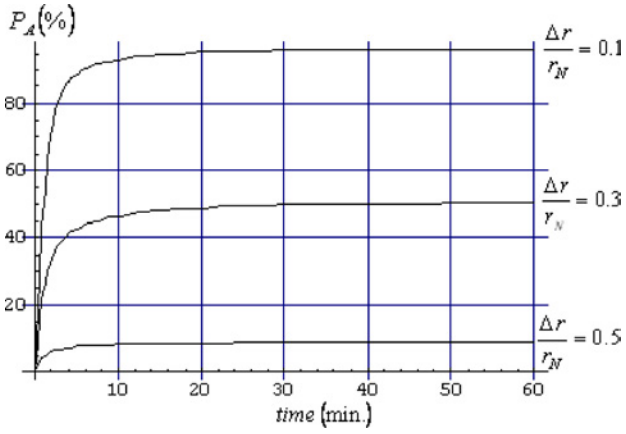

FIGURE 2. Self-stabilization effect during unfolding of *adenylate kinase* for the same temperature history. $\frac{\Delta r_1}{r_{N,1}}$ measures the relative change in size of the unfolded protein.

contact at the distal forearm (DF) location (see Ref.[34] for details).

First, we considered a homogeneous solution of *adenylate kinase* for which the characteristic thermodynamical parameters T_m and ΔH_{un} of the protein are known, $T_{m,1} = 51.8^\circ$C and $\Delta H_{un,1} = 397.48\,\text{kJ mol}^{-1}$.[25] The radius of gyration in the native state is approximated to $r_{N,1} \cong 2\,\text{nm}$.[31] We derived the relative change with temperature of the population distribution in the aggregated state of the protein for various volume occupancies ranging between $15\% \leq f \leq 25\%$. The corresponding thermograms are displayed in Fig. 1. One can observe the tendency of proteins to become thermally more stable with increasing the volume of occupancy (f). Therefore, high protein concentration is a source of crowding. An enhancement of the protein stability at high protein concentration was reported in experimental measurements.[35] We can predict also the level of self-stabilization expected during unfolding of adenylate kinase for a $f = 20\%$ volume occupancy in solution. The self-stabilization occurs due to steric effects induced by the unfolding of a fraction of proteins in solution. As the volume available per unfolded protein is larger than that corresponding to a native protein, this will impose geometrical constraints (volume exclusion) on the proteins in native states, as described above. In Fig. 2 we display the time evolution of the probability distribution in the aggregated state of adenylate kinase for various values of the parameter $\frac{\Delta r_1}{r_{N,1}}$. Measures the relative change in size of the unfolded protein. As we can see, the denaturation of protein is progressively inhibited by conformational change that increases the volume of the protein.

The system of Eqs. (1), taken together with (8), (9), (16) and (19) allow us to analyze the thermal stability of mixtures of proteins. We can examine, for example, how the unfolding and subsequent aggregation of thermally labile proteins influence the stability of those proteins in the mixture which have higher midpoint transitions. For brevity, we consider that the volume fraction of $f = 20\%$ of the solution is occupied by *adenylate kinase* and three other different proteins, *creatine kinase, ATP synthase e* and *cytochrome c*, with equal partitions $x_1 = x_2 = x_3 = x_4$, ($\sum_{j=1}^{4} x_j = 1$). The characteristic physical parameters T_m and ΔH_{un} for the last three proteins, (2) *creatine kinase*, (3) *ATP synthase e* and (4) *cytochrome c* are as follow: $T_{m,2} = 56^\circ$C, $\Delta H_{un,2} = 461\,\text{kJ mol}^{-1}$,[22] $r_{N,2} \cong 5.56\,\text{nm}$,[13] $T_{m,3} = 57.5^\circ$C, $\Delta H_{un,3} = 539.24\,\text{kJ mol}^{-1}$,[36] $r_{N,3} \cong 4.4\,\text{nm}$,[15] $T_{m,4} = 60^\circ$C, $\Delta H_{un,4} = 338.9\,\text{kJ mol}^{-1}$[20] and $r_{N,4} \cong 2\,\text{nm}$,[1] respectively. In Fig. 3(a–c) we display the evolution in time of the denaturation of *creatine kinase*, *ATP synthase e* and *cytochrome c*. The top curves represent always the denaturation in homogeneous solutions, while the bottom curves illustrates the behaviour of the respective protein in the mixture with the other three protein species. Apparently, the steric effects brought by the unfolding of thermolabile proteins, mainly *adenylate kinase*, enhance the stability of all the other protein species (*creatine kinase, ATP synthase e* and *cytochrome c*) in the mixture. Obviously, the stabilization of the protein with the highest melting point transition (*cytochrome c*) is the result of a self-stabilization effect, as discussed above, and contributions from the unfolding of all the other protein species.

Comparison with Experiment

There are some recent reports of experimentally observed crowding and confinement effects on dynamics of proteins. Thus, experiments have shown that proteins encapsulated in small pores in hydrated silica glasses have an enhanced stability when heated.[12] In addition, calorimetric measurements revealed that the melting temperature of *actin* increases by approximately 5°C in the

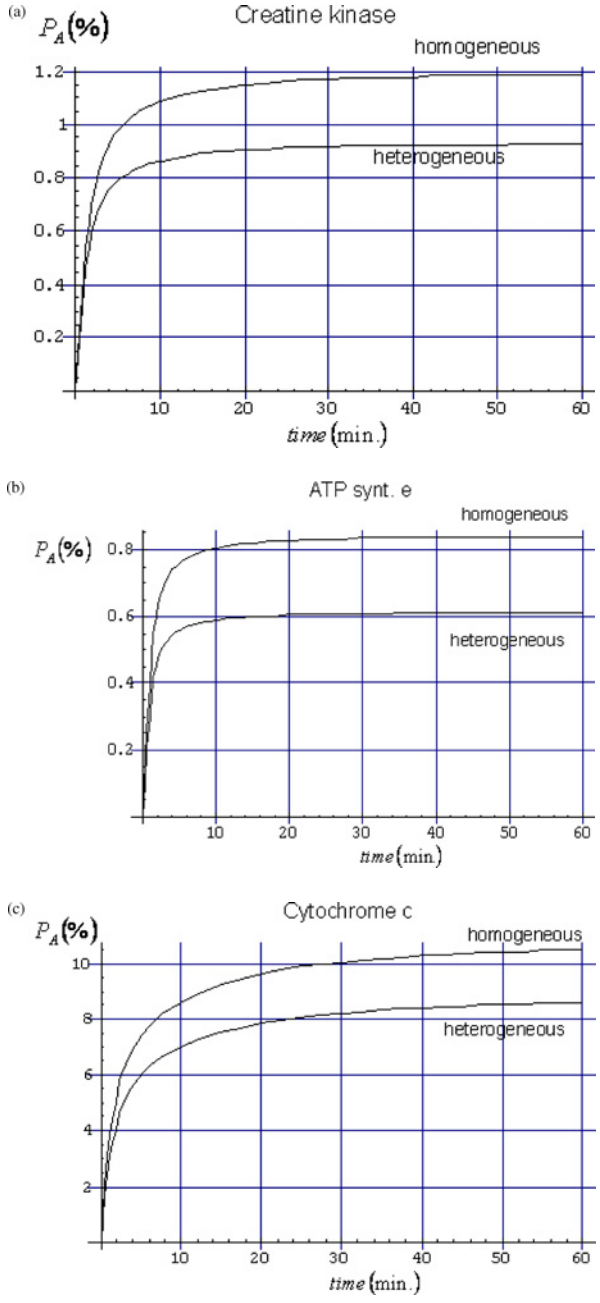

FIGURE 3. The steric effects brought by the unfolding of thermolabile proteins in the mixture enhance the stability of those proteins in the mixture which have higher melting points. The time–temperature course is the same as the one used in Fig. 1. The top curves represent the denturation of a protein in a homogeneous solution while the bottom curves describe the course of denaturation of this protein in a mixture with other three proteins. (a) *creatine kinase*, (b) *ATP synthase e*, (c) *cytochrome c.*

presence of 100 mg/ml PEG-6000, a non-ionic surfactant.[32] It is also well known that collagen molecules embedded within the lattice of a fiber are substantially more thermally stable than the same molecules in dilute solution.[27,33] An increased stability against denaturation in highly occupied volume of homogeneous solutions is reported also in Ref.[35] All these experimental observations support our theoretical predictions.

The investigation of steric effects on individual proteins in mixed protein solutions is complicated by the likely presence of direct intermolecular interactions in addition to excluded volume interactions. Such complications make it to difficult to use standard calorimetric measurements to test our prediction that the unfolding of the most thermolabile proteins will increase the stability of the other proteins in solution. A more detailed analysis, including an adequate experimental protocol, is in progress.

CONCLUSIONS

Within the present paper, we call attention to some theoretical approaches that may help to resolve conceptual ambiguities and enhance quantitative analyses of the kinetic stability of cellular components confronted with the destabilizing effect of irreversible alteration. We provide a basic introductory tutorial to the Lumry–Eyring model theory and describe all the temperature-dependent terms entering the kinetic rates. On this basis, we can examine critically the limitations of using calorimetric data of dilute protein solutions to interpret situations in finite volume occupancies. We pointed out the influence of high-fractional volume occupancy on the rates and equilibria of thermal denaturation taking place in a crowded biological environment. This aspect is analyzed in a quantitative manner. Present results suggest that crowding effects might play the determinant role for the structural stability of the cell when heated. This inference is consistent with experimental observations.[12,19,32,35] A source for a further enhancement of our capabilities to infer the *in vivo* behavior of specific cellular components at high temperatures is the experimental approach proposed by Lepock[18] and Bischof[2] in which the entire cell is monitored. However, to decipher the entire cell thermogram and ascribe the endotherm maxima to specific cellular components can be a difficult task.

Our predictions may apply to fibers and basement membranes in which the increased stabilization by crowding and conformational changes is a general effect. The present approach represents the initial stage in our effort to provide a means for monitoring the evolution of the structural alteration of the cellular components at specific locations of a burn injury. The method can prove also very informative for developing strategies to minimize the tissue damage.

ACKNOWLEDGMENTS

The research presented here has been partly supported by the National Institutes of Health, grants R01 GM61101 (RCL) and R01 GM64757 (RCL), and The Electric Power Research Institute (RCL).

REFERENCES

[1]Akiyama, T., S. Takahashi, T. Kimura, K. Ishimori, I. Morishima, Y. Nishikawa, and T. Fujisawa. Conformational landscape of cytochrome *c* folding studied by microsecond-resolved small-angle X-ray scattering. *Proc. Natl. Acad. Sci. USA* 99:1329–1334, 2002.

[2]Bhowmick, S., D. J. Swanlund, and J. C. Bischof. Supraphysiological thermal injury in dunning AT-1 prostate tumor cells. *ASME J. Biomech. Eng.* 122:51–59, 2000.

[3]Despa, F., and R. S. Berry. Inter-basin Dynamics on multidimensional potential surfaces. I. Escape rates on complex basin surfaces. *J. Chem. Phys.* 115:8274–9278, 2001.

[4]Despa, F., and R. S. Berry. Relaxation dynamics in the presence of unequally spaced attractors along the reaction coordinate. *Eur. Phys. J. D* 16:55–58, 2001.

[5]Despa, F., and R. S. Berry. Inter-basin dynamics on multidimensional potential surfaces. Kinetic traps. *Eur. Phys. J. D* 24:203–206, 2003.

[6]Despa, F., A. Fernàndez, R. S. Berry, Y. Levy, and J. Jortner. Inter-basin motion approach to dynamics of conformationally constrained peptides. *J. Chem. Phys.* 118:5673–5682, 2003.

[7]Despa, F., D. P. Orgill, J. Newalder, and R. C. Lee. The relative thermal stability of tissue macromolecules and cellular structure in burn injury. *Burns* (in press).

[8]Diller, K. R. Analysis of skin burns. In: Heat Transfer in Medicine and Biology: Analyses and Applications, Vol. 2, New York: Plenum, 1985, pp. 85–134.

[9]Diller, K. R. Modeling of bioheat transfer process at high and low temperatures. *Adv. Heat Transfer* 22:157–357, 1992.

[10]Diller K. R., and J. A. Pearce. Issues in modeling thermal alterations in tissues. *Ann. N. Y. Acad. Sci.* 888:153–163, 1999.

[11]Diller K. R., J. W. Valvano, and J. A. Pearce, Bioheat Transfer. In: *The CRC Handbook of Mechanical Engineering, 2nd Edition*, edited by F. Kreith and Y. Goswami. Boca Raton: CRC Press, 2005, 4-282–4-361.

[12]Eggers, D. K., and J. S. Valentine. Crowding and hydration effects on protein conformation: A study with sol–gel encapsulated proteins. *J. Mol. Biol.* 314:911–922, 2001.

[13]Forstner, M., M. Kriechbaum, P. Laggner, and T. Wallimann. Structural changes of creatine kinase upon substrate binding. *Biophys. J.* 75:1016–1023, 1998.

[14]Fulton, A. B. How crowded is the cytoplasm? *Cell* 30:345–347, 1982.

[15]Harada, M., Y. Ito, M. Sato, O. Aono, S. Ohta, and Y. Kagawa, Small-angle X-ray scattering studies of Mg.AT(D)P-induced hexamer to dimer dissociation in the reconstituted alpha 3 beta 3 complex of ATP synthase from thermophilic bacterium PS3. *J. Biol. Chem.* 266:11455–11460, 1991.

[16]Lee, R. C., and R. D. Astumian. The physico-chemical basis for thermal and nonthermal "burn" injury. *Burns* 22:509–519, 1996.

[17]Lee, R. C., D. Zhang, and J. Hannig. Biophysical injury mechanisms in electrical shock trauma. *Annu. Rev. Biomed. Eng.* 2:477–509, 2000.

[18]Lepock, J. R. Cellular effects of hyperthermia: Relevance to the minimum dose for thermal damage. *Int. J. Hyperthermia* 19:252–266, 2003.

[19]Li, J., S. Zhang, and C. Wang. Effects of macromolecular crowding on the refolding of glucose-6phosphate dehydrogenase and protein disulfide isomerase. *J. Biol. Chem.* 37:34396–34401, 2001.

[20]Liggins, J. R., T. P. Lo, G. D. Brayer, and B. T. Nall. Thermal stability of hydrophobic heme pocket variants of oxidized cytochrome *c*. *Protein Sci*. 8:2645–2654, 1999.

[21]Lumry, R., and E. Eyring. Conformation changes in proteins. *J. Phys. Chem.* 58:110–120, 1954.

[22]Lyubarev, A. E., B. I. Kurganov, V. N. Orlov, and H. M. Zhou. Two-state irreversible thermal denaturation of muscle creatine kinase. *Biophys. Chem.* 79:199–204, 1999.

[23]Mackie, J. S., and P. Meares, The diffusion of electrolytes in a cation-exchange resin membrane I. Theoretical. *Proc. R. Soc. Lond.* A 232:498–509, 1955.

[24]Makhatadze, G. I., and P. L. Privalov. Energetics of protein structure. *Adv. Protein Chem.* 47:307–425, 1995.

[25]Mantsch, H. H., J. Reinstein, K. Le Blay, A. M. Gilles and O. Barzu. Structural and functional consequences of amino acid substitutions in the second conserved loop of *Escherichia coli* adenylate kinase. *J. Biol. Chem.* 266:23654–23659, 1991.

[26]Minton, A. P. Effect of a concentrated "inert" macromolecular cosolute on the stability of a globular protein with respect to denaturation by heat and by chaotropes: A statistical-thermodynamic model. *Biophys. J.* 78:101–109, 2000.

[27]Na, G. C. Monomer and oligomer of type I collagen: Molecular properties and fibril assembly. *Biochemistry* 28:7161–7167, 1989.

[28]Orgill, D. P., M. G. Solari, M. S. Barlow, and N. E. O'Connor. A finite-element model predicts thermal damage in cutaneous contact burns. *J. Burn. Care Rehabil.* 19:203–209, 1998.

[29]Plaza del Pino, I. M., B. Ibarra-Molero, and J. M. Sanchez-Ruiz. Lower kinetic limit to protein thermal stability: A proposal regarding protein stability *in vivo* and its relation with misfolding diseases. *Proteins* 40:58–70, 2000.

[30]Poland, D. Free energy distributions in proteins. *Proteins* 45:325–336, 2001.

[31]Russell, P. J., Jr., E. Chinn, A. Williams, C. David-Dimarino, J. P. Taulane, and R. Lopez. Evidence for conformers of rabbit muscle adenylate kinase. *J. Biol. Chem.* 265:11804–11809, 1990.

[32]Tellam, R. L., M. J. Sculley, L. V. Nichol, and P. R. Wills. Influence of poly(ethylene glicol) 6000 on the properties of skeletal-muscle actin. *Biochem. J.* 213:651–659, 1983.

[33]Tiktopulo, E. I., and A. V. Kajava. Denaturation of type I collagen fibrils is an endothermic process accompanied by a noticeable change in the partial heat capacity. *Biochemistry* 37:8147–8152, 1998.

[34]Tropea, B. I., and R. C. Lee. Thermal injury kinetics in electrical trauma, *J. Biomech. Eng.* 114:241–250, 1992.

[35]Waldner, J. C., S. L. Lahr, M. H. Edgell, and G. J. Pielak. Nonideality and protein thermal denaturation, *Biopolymers* 49:471–479, 1999.

[36]Wang, Z.-Y., E. Freire, and R. McCarty. Influence of nucleotide binding site occupancy on the thermal stability of the F1 portion of the chloroplast ATP synthase. *J. Biol. Chem.* 268:20785–20790, 1993.

[37]Wright, N. T., and J. D. Humphrey. Denaturation of collagen via heating: An irreversible rate process, *Annu. Rev. Biomed. Eng.* 4:109–128, 2002.

[38]Yannas, I. V., J. F. Burke, P. L. Gordon, C. Huang, and R. H. Rubenstein. Design of an artificial skin. II. Control of chemical composition. *J. Biomed. Mater. Res.* 12:7–32, 1980.