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REVIEW

Kinetic stability of membrane proteins

F. Luis González Flecha [1]

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Abstract Although membrane proteins constitute an important class of biomolecules involved in key cellular processes, study of the thermodynamic and kinetic stability of their structures is far behind that of soluble proteins. It is known that many membrane proteins become unstable when removed by detergent extraction from the lipid environment. In addition, most of them undergo irreversible denaturation, even under mild experimental conditions. This process was found to be associated with partial unfolding of the polypeptide chain exposing hydrophobic regions to water, and it was proposed that the formation of kinetically trapped conformations could be involved. In this review, we will describe some of the efforts toward understanding the irreversible inactivation of membrane proteins. Furthermore, its modulation by phospholipids, ligands, and temperature will be herein discussed.

Keywords Thermal stability · Irreversible denaturation · Kinetic analysis · Membrane enzymes · P-ATPases · Protein unfolding

Introduction

Soluble and membrane proteins are the two main structural classes of proteins. Membrane proteins constitute a heterogeneous group of proteins, including transporters, receptors, and channels, sharing the characteristic of being embedded into biological membranes. They represent about 25–30% of total proteins codified in known genomes (Almén et al. 2009; Wallin and Von Heijne 1998) and constitute the target of about 70% of current drugs (Yildirim et al. 2007).

Whereas globular proteins are folded around a hydrophobic core surrounded by a water-accessible surface (Creighton 1996), membrane proteins have some of their more hydrophobic residues interacting with phospholipids, thus defining a hydrophobic transmembrane surface in the protein, and a monolayer of lipids with restricted mobility covering it (Marsh 2008). Multidomain membrane proteins constitute a very interesting subgroup of membrane proteins which are involved in essential cellular functions, such as active transport and signal transduction. They contain a membrane-associated region and one or more water-related domains. The synchronized interaction among these regions determines the coupling between cytoplasmic-related biological activities (e.g., molecular recognition, catalysis, etc.) and protein interactions with membrane lipids, which determine membrane-associated biological processes (e.g., transport). As some of these proteins are involved in the movement of hydrophilic species across membranes, the core of the transmembrane region may be hydrophilic, thus allowing the interaction with water-soluble species (Harris and Booth 2012).

As expected, the complex structural organization required for adaptation to the physicochemically heterogeneous membrane–water environment determine important experimental challenges when working with these proteins, so the reported studies on their folding and stability are very few (Fleming 2014; Otzen and Andersen 2013; Roman and González Flecha 2014; White and Wimley 1999).

Two simple models have driven much of the early research on membrane protein folding. In the 1990s, Popot

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✉ F. Luis González Flecha
lgf@qb.ffyb.uba.ar

[1] Universidad de Buenos Aires, CONICET, Laboratorio de Biofisica Molecular, Instituto de Química y Fisicoquímica Biológicas, Buenos Aires, Argentina

and Engelman proposed a thermodynamic model describing the folding of α-helix type membrane proteins according to the following sequence of steps: (i) insertion of the aminoacidic chain into the membrane and folding to form stable α-helices; (ii) association of the α-helices to form an intermediate "bundle"; (iii) global rearrangement of the protein structure and/or attachment of prosthetic groups to reach its functional state (Popot and Engelman 1990, 2000). Besides, the so-called positive-inside rule (von Heijne 1986) generalizes the observation that loops connecting transmembrane helices oriented toward the cytoplasm are rich in lysine and arginine residues. This rule is widely used to predict the topology of the transmembrane region of membrane proteins.

The stability of protein structures is usually defined as the resistance of native folded conformations against their disruption by environmental factors promoting denaturation. This process is usually reversible for many small globular proteins, allowing the thermodynamic characterization of the unfolding process (i.e. the thermodynamic stability). Membrane proteins display a variable degree of irreversibility, so thermodynamic stability was only characterized in very few cases.

Alternatively, protein stability can be evaluated by measuring the time persistence of the native folded conformation (i.e. the kinetic stability). Kinetics does not have the limitation of being restricted to reversible processes in equilibrium, so it is a valuable tool to deal with irreversible processes. In this review, we will discuss some theoretical and practical considerations about the kinetic stability of membrane proteins.

Thermodynamic stability: the two-state reversible model

Experimental studies on protein thermodynamic stability require three conditions to be fulfilled: (i) the protein folding process has to be reversible, so that the protein can be folded and unfolded at equilibrium; (ii) there is an agent capable of perturbing the equilibrium, thus changing the concentrations of native, intermediate, and unfolded species; and (iii) there is a measurable signal able to monitor the perturbation. If these three conditions are met, equilibrium thermodynamic tools allow to determine the Gibbs free energy change for transferring the protein from an ideal one molar solution of the pure folded protein in water to an ideal one molar aqueous solution of the pure unfolded protein in water, i.e., $\Delta G_w°$, which is the accepted form to quantify the structural thermodynamic stability of a given protein (Privalov 1979; Schellman 1987; Tanford 1968).

The simplest protein unfolding process can be described as an elemental chemical reaction where the reactant is the native protein (N) and the product is the unfolded protein (U) (this is known as the two-state reversible model):

$$U \underset{k_U}{\overset{k_F}{\rightleftarrows}} N \tag{1}$$

where k_F and k_U are the folding and unfolding rate coefficients, respectively. At the equilibrium:

$$\frac{[U]_{eq}}{[N]_{eq}} = \frac{f_U}{f_N} = \frac{k_U}{k_F} = \boldsymbol{K}_U = e^{-\frac{\Delta G_w^o}{R \cdot T}} \tag{2}$$

where f_N and f_U are the mole fractions of the native and unfolded protein at equilibrium, respectively, and $\boldsymbol{K}_U$ is the thermodynamic equilibrium constant.

Two main strategies are used for perturbing the folding equilibrium of a given protein: the use of chemical denaturing agents (solvent denaturation) and temperature changes (heat denaturation).

Solvent-induced equilibrium unfolding can be characterized by two parameters: $\Delta G_w°$ and m_{NU} (the slope of the dependence of the free energy change with denaturant concentration). These parameters have to be obtained from some experimental signal (our observable). As a rule, a good signal must be able to be expressed as a linear combination of the concentrations of the species present in the system:

$$S_{total} = f_N \cdot S_N + f_U \cdot S_U \tag{3}$$

where S_N and S_U are the signal values for the native and unfolded states, respectively.

Usually, S_N and S_U (in the pre-transition and post-transition regions), and $\Delta G°$ vary linearly with denaturant concentration (Greene and Pace 1974). Thus, the total signal (S_{total}) will be related to the equilibrium unfolding parameters (Fleming 2014):

$$S_{total} = \frac{S_{N,w} + m_N \cdot [D] + \left(S_{U,w} + m_U \cdot [D]\right) \cdot e^{-\frac{\Delta G_w^o - m_{NU} \cdot [D]}{R \cdot T}}}{\left(1 + e^{-\frac{\Delta G_w^o - m_{NU} \cdot [D]}{R \cdot T}}\right)} \tag{4}$$

$\Delta G_w°$ and m_{NU} values for a given unfolding process can be estimated by fitting Eq. 4 to the experimental data using nonlinear regression methods (Kemmer and Keller 2010).

On the other hand, heat denaturation is a result of the temperature dependence of the unfolding Gibbs free energy (Schellman 1987):

$$\Delta G_w^o = \Delta H_w^o - T \cdot \Delta S_w^o - \Delta Cp_w^o \left[(T_o - T) + T \cdot \ln\left(\frac{T}{T_o}\right) \right] \tag{5}$$

Increasing or decreasing the temperature after reaching a maximal stability produces a decrease in $\Delta G_w°$, which becomes negative for temperatures higher than the so-called

thermal denaturation temperature (T_m) or lower than the cold denaturation temperature (T_c). Convexity is given by the heat capacity change upon unfolding ($\Delta Cp_w°$), which reports the difference between solvent interactions in the folded and unfolded states, and it has been found to correlate with the change in the solvent-accessible surface area (ΔASA) as a protein unfolds (Myers et al. 1995).

Reversible unfolding of membrane proteins

Solvent denaturation has been extensively used to characterize the thermodynamic stability of water-soluble proteins (Schellman 2002). Among various chemical denaturing agents, urea and guanidine hydrochloride (GdnHCl) have proved particularly successful. In a similar way, many β-barrel membrane proteins can be denatured by urea and guanidinium chloride (Buchanan et al. 2012; Burgess et al. 2008; Kleinschmidt 2015). On the contrary, helical membrane proteins are usually resistant to these denaturants, but are prone to be denatured by detergents such as sodium dodecyl sulfate (SDS) (Hong et al. 2009; MacKenzie 2006).

Reversible unfolding has been achieved only for a few membrane proteins, among them bacteriorhodopsin (Curnow and Booth 2007; Faham et al. 2004), diacylglycerol kinase (Lau and Bowie 1997), the KcsA potassium channel (Barrera et al. 2005), the disulfide bond reducing protein DsbB (Otzen 2003), *Archaeoglobus fulgidus* CopA (Roman et al. 2010), rhomboid intramembrane proteases (Panigrahi et al. 2016), and for some outer membrane proteins (Andersen et al. 2012; Danoff and Fleming 2017; Hong and Tamm 2004; Huysmans et al. 2010; Moon and Fleming 2011a; Pocanschi et al. 2013). In a recent work, Di Bartolo et al. (2016) have shown that immobilization of a helical membrane protein on a solid matrix improves unfolding reversibility.

Equilibrium and, in some cases, kinetic characterization of the reversible unfolding of these membrane proteins provides a preliminary picture of this process. Booth and co-workers observed that unfolding free energies and their derivatives with respect to the denaturant concentration (m) were similar to those measured in soluble proteins of comparable size (Booth and Curnow 2009). Furthermore, kinetic studies suggest that the transition state is a loosely organized ensemble of conformations (Schlebach et al. 2014) and its configuration is closer to that of the unfolded state (Curnow and Booth 2009). An in-depth discussion of advances, challenges, and perspectives in reversible membrane protein folding can be found in recent reviews by Fleming (2014), Harris et al. (2017), Otzen and Andersen (2013), and Roman and González Flecha 2014, and references therein.

Single molecule methods have emerged as a new tool to investigate reversible membrane protein folding (Jefferson et al. 2017). They have the important advantage of working with very low protein concentrations, thus overcoming the aggregation problem. It is expected that the combination of bulk and single molecule studies shed new light on the energetics and mechanism of membrane protein folding.

Kinetic stability: the two-state irreversible model

The simplest irreversible protein denaturation model involves two steps: (a) reversible unfolding of the native protein; (b) irreversible alteration of the unfolded protein to yield a final irreversibly denatured state (D):

$$N \underset{k_F}{\overset{k_U}{\rightleftarrows}} U \xrightarrow{k_{irrev}} D \tag{6}$$

This model is usually known as the Lumry–Eyring mechanism (Lumry and Eyring 1954; Sanchez-Ruiz 2010). According to the relative values of the kinetic coefficients, two main scenarios can be envisaged (Sanchez-Ruiz 1992).

If $k_{irrev} \ll k_F$, a rapid equilibrium between N and U will be established. In this condition, the concentration of D will follow a first-order kinetics with an apparent kinetic coefficient $k_{app} = \boldsymbol{K}_U.k_{irrev}$. Of particular interest is the case when $\boldsymbol{K}_U = k_u/k_f \ll 1$, i.e., when working below the melting temperature. In this situation, the concentration of U will be very low, and it can be considered that N and D are the only species in the system.

If $k_{irrev} \gg k_F$, U will be converted to D as soon as it is formed from N. In this condition, the concentration of D will follow a first-order kinetics with an apparent kinetic coefficient $k_{app} = k_U$.

In both cases, the irreversible transition can be described by a simplified model denoted as the two-state irreversible model:

$$N \xrightarrow{k_{app}} D \tag{7}$$

The fact that Lumry–Eyring mechanisms reduce to a phenomenological two-state kinetic process has represented a significant advance in the experimental study of irreversible denaturation processes (Sanchez-Ruiz 2010).

The structural basis of protein kinetic stability are not yet well established. Colón and co-workers found a relation between kinetically trapped native conformations and their resistance to denaturation by SDS, and suggested that oligomers, β-sheet structures, and topologies with many long-range contacts could be related with high kinetic stability (Broom et al. 2015; Manning and Colón 2004). Besides, Marqusee and co-workers found that high kinetic stability is related to unusual resistance to proteolysis; however, they do not find any common structural features that account for

proteolytic resistance (Park et al. 2007). Several works have pointed out the importance of ionic, hydrophobic, π–π, and cation–π interactions in stabilizing proteins in the high-temperature regime, and conclude that the involved mechanisms cannot be fully rationalized on the basis of the sum of individual contributions but require a global analysis in terms of interaction networks (Karshikoff et al. 2015; Pucci and Rooman 2017).

Measuring the kinetic stability of membrane proteins

As was previously mentioned, most membrane proteins undergo irreversible thermal denaturation, even under moderate environmental conditions. In these cases, it is possible to evaluate protein stability by kinetic methods based on quantifying the time persistence of the native folded conformation. This can be monitored through measurable signals, such as enzyme activity (or another measure for functionality), molar ellipticity in the far UV (Powl et al. 2012; Roman et al. 2011), or the intensity of emitted fluorescence from either the intrinsic Trp residues (Moon and Fleming 2011b) or given by an external fluorescent probe, such as 1-anilinonaphthalene-8-sulphonate (Cattoni et al. 2009; Dodes Traian et al. 2012) or fluorescent ligand analogues (Placenti et al. 2017).

The time course of these signals provides a measure of the kinetic stability of the protein. As discussed in the previous section, the observed signal usually changes with the incubation time following a single exponential function at a given temperature:

$$S_t = (S_0 - S_\infty)\cdot e^{-k_{app}\cdot t} + S_\infty \quad (8)$$

where S_t is the signal value at incubation time (t), S_0 and S_∞ are the values of the signal at $t = 0$ or $t \to \infty$ respectively, and k_{app} is the apparent rate coefficient for the irreversible formation of D.

The observed monoexponential behavior suggests a two-state process involving only two membrane protein ensembles, one characterized by the S_0 value of the signal (corresponding to N) and the other by an S_∞ value (corresponding to D). The two-state character of the observed transition can be confirmed if different signals referring to different protein properties follow the same monoexponential function.

This was the case of several P-ATPases for which the kinetic coefficients describing the time course of the structural changes detected by Trp, ANS and eosin fluorescence, circular dichroism, and ion occlusion capacity are not statistically different from that corresponding to the inactivation process (Cattoni et al. 2008; Kaufman et al. 2012; Levi et al. 2000; Placenti et al. 2017), indicating that changes in the enzyme activity and secondary and tertiary structure are all part of the same global change produced by incubation at high temperatures.

Several other experimental strategies have been used for evaluating the kinetic stability of membrane proteins. Among these, differential scanning calorimetry (Galisteo and Sanchez-Ruiz 1993; Sedlák et al. 2014) has provided the most valuable information on the energetics of the involved processes (for a good discussion, see Sanchez-Ruiz 1992, 2010). In addition, some new approaches, such as pulse proteolysis (Di Bartolo et al. 2016; Schlebach et al. 2011) and steric trapping (Jefferson et al. 2013), are becoming promising tools to explore complementary aspects of protein kinetic stability.

The thermally induced membrane protein denatured state

The reversible denatured state of soluble proteins has been characterized as an ensemble of multiple conformations with a very low content of secondary and tertiary structure (Shortle and Ackerman 2001). Contrasting with the observed parallelism in the unfolding energetics between membrane and soluble proteins, helical membrane proteins seem to retain significant secondary structure in the unfolded states (Booth and Curnow 2009; Roman et al. 2010). This has lead to redefine the folding equation for membrane proteins as a transition from the native state to an "intramembranous unfolded state", defined as a non-native (and not random-coil like) state embedded in the bilayer (Fleming 2014).

Irreversible inactivation has been extensively characterized for soluble proteins and, in many cases, it is due to either covalent modifications (hydrolysis of peptide bonds, side-chain oxidation, destruction of disulfide bonds, formation of incorrect disulfide bonds, deamidation, racemization, etc.) or non-covalent changes leading to aggregation or the formation of kinetically trapped conformations (Ahern and Klibanov 1985; Nury and Meunier 1990; Yan et al. 2004).

A precise characterization of the temperature-induced denatured state of membrane proteins was performed by Bowie and co-workers. Analyzing the irreversible inactivation mechanism of the membrane protein diacylglycerol kinase in detergent micelles, the authors found that thermal inactivation is not associated with any of the covalent modifications found in soluble proteins. In addition, the protein was more stable at higher protein concentrations, contrary to that expected if aggregation was the primary cause of inactivation. Thus, they proposed that other non-covalent changes, such as the formation of kinetically trapped conformations, could be involved (Zhou et al. 2001).

In P-type ATPases, it was found that the decrease in enzyme activity is paralleled by large changes in secondary and tertiary structure (Cattoni et al. 2008; Kaufman et al. 2012; Levi et al. 2000; Placenti et al. 2017). The irreversible loss of ATPase activity was not due to either degradation or formation

of SDS-stable aggregates of the protein. On the other hand, no change in the distribution of oligomeric forms was found after incubation at high temperatures, suggesting that the quaternary structure is not significantly affected by the thermal inactivation process. Despite this, a careful study using small-angle X-ray scattering demonstrated that spontaneous formation of low amounts of high-order oligomeric species occur even at low temperatures (Yoneda et al. 2016).

Circular dichroism analysis in the far UV region showed that the inactivated state still conserves an important secondary structure content (Cattoni et al. 2008), and comparison between the resulting spectra and those of denatured soluble proteins (Sreerama et al. 2000) and native membrane proteins (Park et al. 1992) suggest that the major change occurs in the soluble domains. Indeed, the persistence of ANS fluorescence when extrapolated to infinite incubation times at high temperatures indicates the conservation of membrane-associated hydrophobic regions. Thus, it was postulated that the structural changes associated to the inactivation process mainly reflect the disorganization of the cytoplasmic regions of these multidomain membrane enzymes, together with a slight unfolding of the transmembrane domain.

A very nice study on the thermal unfolding of NaChBac sodium channel using synchrotron radiation circular dichroism showed that the protein undergoes an initial irreversible unfolding of the extramembranous C-terminal helical region, which was accompanied by a reversible unfolding of a small number of helical residues in the transmembrane domain. At very high temperatures, this process is followed by the irreversible unfolding of a limited number of additional transmembrane helical residues (Powl et al. 2012).

Modulating the kinetic stability of membrane proteins by phospholipids

It is well known that the thermal stability of membrane proteins is higher when they are inserted in their native membrane than when are solubilized and reconstituted in artificial membrane-like environments.

The modulation of membrane protein stability by phospholipids can be explored using model systems composed of membrane proteins reconstituted in mixed micelles of phospholipids and detergents. In this analysis, mixed micelles are considered as a thermodynamically "separate" phase (Tanford 1980), whose composition is defined by the mole fractions of its components:

$$X_{\mathrm{i,mic}} = \frac{\mathrm{n_i}}{\mathrm{n_{PL}} + \mathrm{n_{det}} + \mathrm{n_{MP}}} \cong \frac{\mathrm{n_i}}{\mathrm{n_{PL}} + \mathrm{n_{det}}} \tag{9}$$

where $X_{\mathrm{i,mic}}$ is the mole fraction of the *i* component of the micelles [*i* may be phospholipid (PL), detergent (det), or membrane protein (MP)] and n refers to the total amount of substance (number of moles). In all cases, $\mathrm{n_{MP}} \ll \mathrm{n_{PL}}$, thus validating the approximation made in Eq. 9.

The influence of the relative concentration of phospholipid and detergent on the thermal stability of membrane proteins can be studied using two alternative experimental designs: either the concentration of detergent is kept constant while the amount of added phospholipids is increased, or vice versa. Comparison between these kinds of experiments performed using the plasma membrane calcium pump as a model, showed that similar values of k_{app} were obtained when the mole fractions of the phospholipids in the micelles were similar, even if the phospholipid concentrations in the whole system were different (Levi et al. 2000). This indicates that the effect of phospholipids and detergent on membrane protein stability is related to the micellar phase composition but not to the concentration of micelles in the system.

To give account of this effect, it was considered that membrane proteins reconstituted in the micellar form has its hydrophobic transmembrane surface always covered by bound amphiphiles. The quantitative description of the association of two amphiphiles competing for the hydrophobic transmembrane surface of the protein (Levi et al. 2003) is given by:

$$\theta_{\mathrm{PL}} = \frac{K_{\mathrm{ex}} \cdot X_{\mathrm{PL,mic}}}{X^{\beta}_{\mathrm{det,mic}} + K_{\mathrm{ex}} \cdot X_{\mathrm{PL,mic}}} \tag{10}$$

where θ_{PL} is the fractional coverage of this surface by phospholipid molecules; $X_{\mathrm{PL,mic}}$ and $X_{\mathrm{det,mic}}$ are the mole fractions of phospholipid and detergent in the micelles, respectively; K_{ex} is the equilibrium constant for the exchange between detergent and phospholipids molecules at the hydrophobic transmembrane surface of the protein; and β is the stoichiometric exchange coefficient.

Levi et al. (2000) demonstrate that k_{app} increases steeply when $X_{\mathrm{det,mic}}$ approaches 1 ($\theta_{\mathrm{PL}} \rightarrow 0$) and becomes minimal when $X_{\mathrm{PL,mic}}$ asymptotically approaches 1. This dependence can be described by a hyperbolic function of θ_{PL}:

$$k_{\mathrm{app}} = \frac{k_{\mathrm{min}}}{\theta_{\mathrm{PL}}} \tag{11}$$

where k_{min} represents the k_{i} value for $\theta_{\mathrm{PL}} = 1$. Substituting θ_{PL} with Eq. 10 gives:

$$k_{\mathrm{app}} = k_{\mathrm{min}} \cdot \left[\frac{X^{\beta}_{\mathrm{det,mic}}}{K_{\mathrm{ex}} \cdot X_{\mathrm{PL,mic}}} + 1 \right] \tag{12}$$

In this model, detergent and phospholipid molecules are in a dynamic equilibrium competing for the hydrophobic transmembrane sites on the protein, i.e., as the phospholipid concentration in the micellar phase increases, detergent molecules are displaced by lipid ones. When the immobilized boundary layer is predominantly composed by phospholipids, the

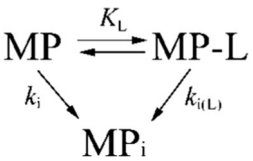
inactivation rate coefficient is minimum (k_{min}) and, therefore, the protein thermal stability (and protein lifetime) is maximal.

For all the systems assayed, this maximal stability was attained when about 80% of the transmembrane protein surface is covered by phospholipid molecules. In addition, this limit value was similar to that obtained for the membrane protein reconstituted in liposomes with the same lipid composition (Levi et al. 2000), but lower than that measured for the enzyme in the native membrane. The value characterizing maximal stability (k_{min}) was found to be dependent on the type of phospholipids included in the mixed micelles, and was also affected when phospholipids undergo chemical modifications, such as non-enzymatic glycation of phosphatidylethanolamine, a common alteration in uncontrolled diabetes mellitus (Levi et al. 2008).

Albert and co-workers performed a careful study of the kinetic stability of rhodopsin by disrupting the disk membrane using detergent (Corley et al. 2011). In this work, the authors found, in addition to the increase of the kinetic coefficient for thermal inactivation when increasing the detergent concentration, that the activation energy of this process is dramatically lowered when the bilayer is disrupted, and remains constant with further detergent addition to the mixed micelles. This result reinforces the idea that the intact native bilayer is critical for the kinetic stability of membrane proteins.

Ligand effects on membrane protein kinetic stability

Protein–ligand interactions play a key role in determining protein structure, stability, and function. The effects of ligand binding on membrane protein kinetic stability may be analyzed considering that both ligand-free and ligand-bound forms of the protein inactivates through a simple irreversible step. Considering that the native membrane protein can bind stoichiometrically a ligand L, then:



where K_L is the corresponding dissociation constant, and k_i and $k_{i(L)}$ are the inactivation rate coefficients for the species MP and MP-L, respectively.

From this model, and assuming that ligand binding is in rapid equilibrium, we conclude that the concentration of native protein decreases following a single exponential function of the incubation time, with an apparent thermal inactivation rate coefficient (k_{app}) being a linear combination of k_i and $k_{i(L)}$ (Placenti et al. 2017):

$$k_{app}\frac{[MP]}{[MP]_{total}}k_i + \frac{[MP{-}L]}{[MP]_{total}}k_{i(L)} \tag{14}$$

In this equation, $[MP]/[MP]_{total}$ and $[MP\text{-}L]/[MP]_{total}$ are the fractions of free and ligand-bound membrane protein, respectively. It can be observed that, in the absence of ligand, k_{app} will be equal to k_i, and when all the ligand sites are occupied, k_{app} will be $k_{i(L)}$. Introducing the equilibrium constant $K_L = [MP]\,[L]/[MP\text{-}L]$:

$$k_{app} = \frac{k_i \cdot K_L + k_{i(L)} \cdot [L]}{K_L + [L]} \tag{15}$$

It can be seen that the change in k_{app} with ligand concentration follows a hyperbolic function of [L] from k_i to $k_{i(L)}$. Remarkably, the $K_{0.5}$ of this hyperbole corresponds to the thermodynamic dissociation constant for the ligand (K_L).

This model was successfully used to explain the effects of several ligands on the Na^+,K^+-ATPase thermal stability (Kaufman et al. 2012; Placenti et al. 2017).

When several ligands bind simultaneously to the protein, the dependence of k_{app} with the concentration of each ligand can show a more complex behavior. This is the case, for example, of the binding of K^+ and ATP to the Na^+,K^+-ATPase (Placenti et al. 2017). To find a model able to describe the experimental results, the authors followed the procedure described by González-Lebrero et al. (2002). In a first step, the concentration of one ligand (e.g., ATP) is kept constant at selected values, and "empirical" equations (quotients of two polynomial functions of the concentration of the other ligand, i.e., K^+) were fitted to the experimental data. Then, the minimal degree of the polynomials giving the best fit was selected. The form of this minimal equation and the dependence of the fitting parameters with the concentration of ATP indicated that six enzyme species are involved in the inactivation pathway. In the second step, a detailed scheme of inactivation is proposed, where each species has its own inactivation rate coefficient and the inactivation process is, in all cases, irreversible. Fitting this model to the global set of experimental data allows to determine the kinetic stability of each of the species involved. Following this procedure, it was determined that the free form of the enzyme and that with one molecule of ATP and K^+ bound are the less stable conformations of the Na^+,K^+-ATPase, whereas the enzyme with two K^+ bound is the more stable one (Placenti et al. 2017).

A particular case of ligand binding occurs when the same protein acts as a ligand, i.e., when oligomerization equilibria are occurring. Levi et al. have explored this process in the plasma membrane calcium pump following a procedure

similar to that previously described but analyzing the apparent coefficient for thermal inactivation as a function of membrane protein concentration. The obtained results showed that thermal inactivation of the calcium pump mainly occurs through monomer denaturation, suggesting that dimerization could constitute a mechanism of self-protection against spontaneous inactivation (Levi et al. 2002).

Dependence on temperature: enthalpic and entropic contributions to the transition state in membrane protein kinetic stability

The temperature dependence of the inactivation rate coefficients can be described by the Arrhenius equation (Laidler and King 1983):

$$\ln\left(\frac{k_{app}}{k_o}\right) = \ln A - \frac{E_a}{R \cdot T} \tag{16}$$

where A is the pre-exponential factor, E_a is the activation energy for thermal inactivation, R is the gas constant, T is the absolute temperature, and k_o is a reference rate coefficient.

Linear Arrhenius plots indicate a common mechanism for thermal inactivation in the explored temperature range. On the contrary, non-linear Arrhenius plots can be found when aggregation becomes a primary cause of inactivation (Wang and Roberts 2013,) generally at the upper limit of the temperature ranges.

Gursky and co-workers explored the physical origin of free energy barriers in the thermal unfolding of lipoproteins and concluded that large activation energy values result from the transient disruption of multiple protein–lipid and lipid–lipid packing interactions (Gursky 2015).

As was discussed previously, the activation energy provides valuable additional information about the thermal stability of membrane proteins (Corley et al. 2011). In this way, a complete characterization of membrane protein kinetic stability would have to include both the analysis of the kinetic coefficients at a given temperature and the activation energy of the thermal inactivation process.

A deeper analysis of the temperature dependence of inactivation rate coefficients can be performed in terms of the transition state theory. In a viscous solvent, the variation of k_{app} with temperature can be described by a Kramers-like model (Hänggi et al. 1990; Kramers 1940) as follows:

$$k_{app} = \nu \cdot \frac{\eta_o}{\eta_T} \cdot e^{-\frac{\Delta G^{\ddagger}}{R \cdot T}} \tag{17}$$

where η is the medium viscosity either at a given reference temperature (η_o) or at the preincubation temperature (η_T), $\Delta G^{\ddagger}$ is the activation free energy, and the frequency coefficient ν can be seen as the k value for a hypothetical barrierless process at the reference temperature. The value of ν cannot be obtained from the experimental data, so it is usually fixed at a consensus value ($10^6\ s^{-1}$), which is considered reasonable for unfolding transitions (Torrent et al. 2008; Zhu et al. 2003).

Given that $\Delta G^{\ddagger}$ can be expressed in terms of the activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$), the variation of k_{app} with temperature will be:

$$k_{app} = \nu \cdot \frac{\eta_o}{\eta_T} \cdot e^{-\frac{\Delta H^{\ddagger} - T \cdot \Delta S^{\ddagger}}{R \cdot T}} \tag{18}$$

This analysis allows to characterize the activation energy barrier in terms of its enthalpic and entropic components. In the Na^+,K^+-ATPase, the $\Delta G^{\ddagger}$ value measured in the absence of ligands (56 kJ/mol) arises from large positive differences in enthalpic (476 kJ/mol) and entropic (420 kJ/mol) contributions (Kaufman et al. 2012). Different enzyme ligands (Na^+, K^+, Mg^{2+}, ATP) produced a slight change in the activation free energy, which is positive for K^+, Mg^{2+}, and ATP (Placenti et al. 2017) and negative for Na^+ (Kaufman et al. 2012). The destabilizing effect of Na^+ was explained because this cation shifted the conformational equilibrium of the enzyme toward a less stable conformation, denoted as E_1 (Kaufman et al. 2012; Yoneda et al. 2013). Conversely, maximal $\Delta G^{\ddagger}$ was observed in the presence of a saturating concentration of K^+, indicating that enzyme species formed in this condition (with two K^+ occluded inside the transmembrane domain) is the most stable one. Enzyme inactivation was characterized in all these cases by high and positive values of the enthalpic and entropic components of the barrier, suggesting a transition state configuration far away from the native state.

Concluding remarks

In this work, we summarized some recent and classical kinetic studies on the irreversible denaturation of membrane proteins. The denaturation process is often well described by a single exponential function of the incubation time. Interestingly, the spectroscopic signals that report on secondary and tertiary structure, and the loss of biological activity follow the same exponential function, in spite of the complexity of the membrane protein structure. This suggests that all the observable signals account for a global and concerted conformational change produced by the incubation at high temperatures.

We have also shown that, when a rapid equilibrium is established before the irreversible step, the dependence of the observed kinetic coefficient with the concentration of the species affecting the equilibrium allow to determine the thermodynamic equilibrium constants involved. This equilibrium may correspond to the unfolding equilibrium, an oligomerization equilibrium, or binding at equilibrium of specific ligands. In this scenario, studies of membrane protein kinetic stability

become an alternative tool to explore some functionally relevant properties.

In recent years, new and exciting advances are being made in this field. Both experimental and computational new approaches are shedding light on hot topics such as the evolution of protein stability (Romero-Romero et al. 2016) and the correlations between protein biological activities, kinetic stability, and molecular flexibility (Quezada et al. 2017). These developments are mainly carried out using soluble proteins as a model. It is expected that advancement in the study of membrane proteins and the development of more powerful research tools will move forward our understanding of mechanisms underlying the adaptation of cellular functions to extreme temperatures in the near future.

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Compliance with ethical standards

Conflict of interest F. Luis González Flecha declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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