Protein Conformations

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Real-Time Monitoring of Protein Conformational Dynamics in Solution Using Kinetic Capillary Electrophoresis**

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Conformational changes represent an important means of regulating protein biological function within the complex environments found in living systems.[1,2] The development and application of techniques able to detect these conformational changes have therefore become a vital area of biophysical research. To date, the most commonly applied methods include nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography (XRC), small-angle X-ray scattering (SAXS), Förster resonance energy transfer (FRET), and electron paramagnetic resonance (EPR) spectroscopy.[3–10]

Generally speaking, the most widely employed method is X-ray crystallography. However, this method provides only a static picture of one protein conformation under certain conditions, whereas NMR, EPR, and fluorescence spectroscopy can probe dynamic conformational changes under more physiologically relevant conditions. NMR spectroscopy can provide results relating to protein conformational mobility in solution and with atomic resolution, but this technique is generally limited to smaller proteins (<25 kDa). Alternatively, fluorescence-based methods (including FRET) can measure the relative distance between intrinsically fluorescent residues or fluorescent labels, related to protein conformational changes. However, the fluorescent labelling of a protein may affect its ability to undergo a given conformational change.

Kinetic capillary electrophoresis (KCE) has emerged as a powerful bioanalytical technique for monitoring a wide range of biomolecular interactions during electrophoretic separation, including the measurement of rate and equilibrium constants associated with protein–ligand binding.[11,12] The power of capillary electrophoresis (CE) has been illustrated through its ability to differentiate between the folded and unfolded states of a protein suggesting it may also be able to monitor, in real-time, the ability of a native, unlabelled protein to undergo large-scale conformational changes.[13,14] This method is rapid, requires little material, and can easily accommodate the presence of allosteric regulators.

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Recently, it has been shown that human tissue transglutaminase (TG2) undergoes large-scale tertiary structural changes related to the regulation of its activity. The binding of guanidine di- or triphosphate (GDP/GTP) inactivates the enzyme in a compact "closed" conformation while Ca^{2+} binding activates the enzyme in the form of an extended "open" conformation.[15–17] TG2 is a structurally and functionally complex protein that has been suggested to function as a cytosolic scaffold protein[18] in addition to its role as a calcium-dependent catalyst capable of cross-linking proteins through transamidation of protein-bound glutamine and lysine side chains.[19–21] Furthermore, unregulated TG2 activities have been implicated in a number of physiological disorders such as Huntington's disease,[22,23] Alzheimer's disease,[24,25] Celiac disease,[26] and in cancer metastasis[27–29] underlining the potential of the enzyme as a therapeutic target.

TG2 comprises four structural domains: an N-terminal β-sandwich, a core domain containing the transamidase active-site catalytic triad (Cys277, His335, and Asp358), and two β-barrels. In its GDP-bound state, the compact structure adopted by TG2 offers restricted accessibility to its active site, in sharp contrast to the extended or open conformational form in which it was crystallized following its calcium-dependent reaction with an irreversible inhibitor.[15,30] Correlation of structural and kinetic data suggests that the open conformational form of TG2 represents the active form of the enzyme, capable of binding the acyl donor substrate.[30–32] Several potential calcium binding sites have been tentatively identified,[39] whereas the nucleotide binding pocket of TG2 associated with GTP/GDP binding has been shown by X-ray crystallography to include residues 476–482 and 580–583 of the first and last strand of β-barrel 1.[15]

In this work we demonstrate the power of using KCE to monitor the large-scale, ligand-induced conformational changes associated with the regulation of human TG2 activity. This represents an important step in assigning functional capacity to conformers of TG2 and establish KCE as a real-time method for studying protein dynamics and function in general.

Conceptually, the method is illustrated in Figure 1. Given the dramatic structural differences between the two conformational forms of TG2, we reasoned that the conformers may be separable by KCE, if their interconversion is slow relative to the separation time between two protein forms. This is illustrated in Figure 1 A, where the structurally larger open form migrates faster than the more compact closed form, resulting in the observation of two quasi-separated peaks. Furthermore, this method would allow the dynamic confor-

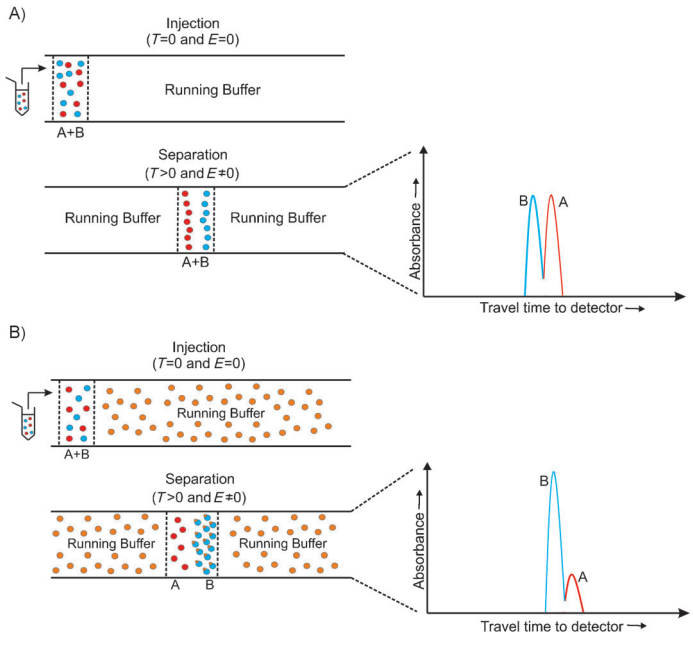


Figure 1. Kinetic capillary electrophoresis experiment. The open conformation of TG2 is shown as blue spheres, while the closed conformation is represented by red spheres. A) A mixture of the two conformational forms of TG2 is injected into the capillary as a short plug. Application of an electric field ($E>0\,V\,cm^{-1}$) results in the migration and separation of the two forms into two fractions. B) Injection and separation of a TG2 sample in running buffer supplemented with a conformational regulator (orange spheres) results in the observation of an altered conformational distribution (T = travel time).

mational equilibrium of TG2 to be probed through supplementation of the electrophoretic running buffer with known conformational regulators of TG2 (Figure 1B). The ability to regulate TG2 conformation during separation would allow a unique kinetic analysis of the protein/regulator interaction.

Human TG2 was expressed and its purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; see the Supporting Information). Analysis of purified TG2 by KCE revealed the presence of two peaks, separated by an approximately 1.5-minute difference in electrophoretic migration times (Figure 2A). This observation is in excellent agreement with earlier studies wherein two forms of TG2 with different electrophoretic mobilities were observed by native polyacrylamide gel electrophoresis (nPAGE) and assigned as the open and closed conformations.[30] In the present study, both peaks eluting from the KCE were confirmed to be purified TG2 by electrospray ionization mass spectrometry, and neither peak corresponds to denatured TG2 (see the Supporting Information). These results suggest that the two peaks correspond to natively folded conformers of TG2, or rather, to two families of closely related conformers. Just as it is naive to assume that TG2 exists only in the two conformations that have been crystallized, it is important to note that each peak observed by KCE may represent a number of similar conformers that migrate together. Minor or rapid conformational changes occurring on the microsecond to millisecond time scale, such as those that would occur during a catalytic cycle of the enzyme, would only lead to slight broadening of each peak corresponding to a conformer family.

This suggests that raising the temperature should increase the rate of interconversion between the conformer families and may lead to coalescence of the peaks. To test this hypothesis, purified TG2 was analyzed by CE from 15 to 50°C through modulation of the capillary running buffer temperature (see the Supporting Information). Ramping of the capillary buffer temperature resulted in the broadening of the two peaks and their coalescence into one peak having an averaged elution time presumably due to conformers that interconvert rapidly at higher temperatures.

We then sought to assign each peak observed by KCE to a known TG2 conformation. To this end, the equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM) method was employed, in which a plug of protein is injected into a capillary prefilled with running buffer containing the known conformational effector.[33] This method is based on the affinity capillary electrophoresis (ACE) approach reported by Whitesides and coworkers where a near-equilibrium is maintained between the interacting species, in this case protein and conformational regulator, during the CE run.[34] KCE separation of TG2 in running buffer supplemented with 20 μM guano-

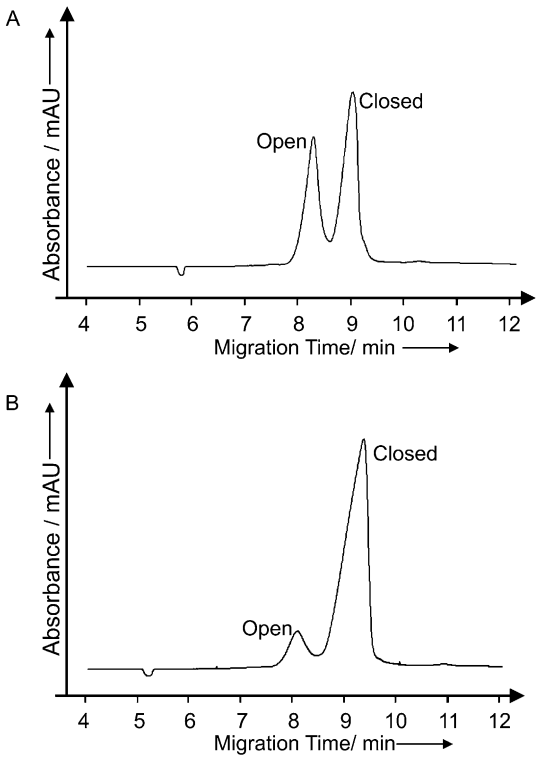


Figure 2. Capillary electropherograms of TG2 in 12.5 mM tris-acetate (pH 8.3). A) KCE separation in the absence of known allosteric regulators (GDP or calcium) showing the protein in the open and closed conformational forms (see labelled peaks). B) KCE separation in the presence of 20 μM GDP supplemented in the running buffer, showing a distinct shift in the conformational distribution from "open" to "closed".

sine diphosphate (GDP) resulted in a shift in conformational distribution in favor of the slower eluting peak (see Figure 2B). The closed conformation was assigned to this peak, according to several lines of evidence. Most significantly, GDP was found to be bound in the X-ray structure of the closed conformation.[35] Furthermore, early SAXS results also suggest that TG2 adopts a more compact conformation in the presence of GDP.[31] Moreover, the intensity of the faster eluting band observed by nPAGE was found to increase upon addition of GDP to the running buffer.[30,32] The fact that the more compact conformation elutes more slowly than the open conformer in CE is not surprising; the open conformation could easily expose more charged residues to the solvent and be subject to greater electro-osmotic force. Finally, when we incubated TG2 with NC9, an irreversible inhibitor developed previously in our group[36] that has been shown to favor a more extended conformation,[37] the intensity of the faster eluting peak increased at the expense of the slower eluting peak (Figure S6 in the Supporting Information).

Although no X-ray structures of TG2 have included bound calcium, previous SAXS, fluorescence, and dynamic CD studies have all suggested that the enzyme adopts a more open conformation in the presence of Ca^{2+}.[17,31,38] Taking into account our observations described above, we predicted that we should be able to increase the proportion of the slower[5c,13a] eluting CE peak by adding increasing amounts of divalent calcium to the running buffer. Our initial observation of two conformational peaks in the absence of added Ca^{2+} suggests that trace amounts of calcium are co-purified with TG2, as has been shown in the past.[39] To minimize the concentration of co-purified calcium, ethylene glycol tetraacetic acid (EGTA), a divalent metal ion chelator having a strong affinity for calcium, was added to the buffers used during the purification of recombinant TG2. This resulted in a significant decrease of the intensity of the faster eluting peak, with a concomitant increase in the intensity of the slower eluting peak (Figure 3). Moreover, increasing the concentration of calcium chloride in the running buffer up to 150 μM resulted in a striking shift of the relative intensity of the faster eluting peak, consistent with displacement of the conformational equilibrium to the active, open conformation (Figure 3). Admittedly the concentration range of Ca^{2+} ions used in the present study is lower than those frequently used in TG2 activity assays, because millimolar concentrations of Ca^{2+} ions resulted in severe broadening and suppression of the protein KCE signal. However, while this may represent a limitation of the experimental method, a recent study has confirmed that TG2 is active at physiological concentrations around 100 μM Ca^{2+} ions,[40] suggesting that the titration range employed herein represents a valid method for probing relevant TG2 behavior. In Figure 3 it can also be noted that adding a salt such as calcium chloride to the running buffer has the general effect of broadening peaks and lengthening migration times. This general salt effect was also observed when magnesium chloride was added to the running buffer instead of calcium chloride; however, adding Mg^{2+} ions up to 150 μM does not have a significant effect on the proportion of the conformers (see the Supporting Information). This important control experiment suggests that the effect of

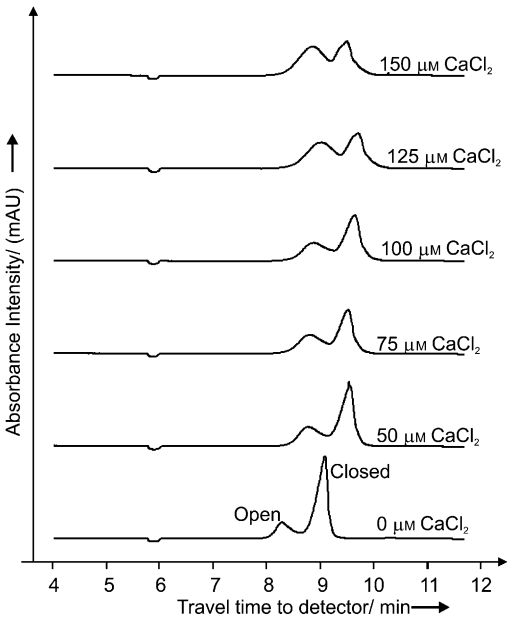
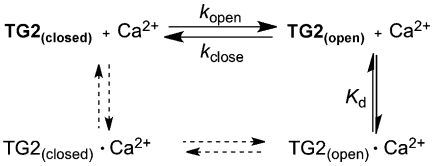


Figure 3. KCE of TG2 showing the influence of increasing concentrations (50 to 150 μM) of calcium chloride on the equilibrium distribution of the open and closed conformations.

added calcium is due to specific ligand binding rather than a general medium effect.

The relative intensities of the peaks assigned to the open and closed forms of TG2 were then analyzed as a function of added calcium concentration, using a previously developed method for studying the kinetics of biomolecular interactions at equilibrium[41] (see the Supporting Information). Since little is known regarding the microscopic details of TG2 opening and calcium binding, we cannot exclude the possible existence of a closed, calcium-bound form of TG2 that can either lose calcium or change conformation by the dashed arrows of Scheme 1. However, for simplicity, fitting was



Scheme 1. Kinetic and thermodynamic parameters for TG2 ligand binding.

performed using the solid arrows of this scheme, based on the species observed crystallographically, shown in bold. This fitting yielded a value of (38 ± 9) μM for the dissociation constant (K_d) of calcium binding, and values of $(138 \pm 27) \times 10^{-3}$ min^{-1} and $(49.8 \pm 1.5) \times 10^{-3}$ min^{-1} were determined for k_{open} and k_{close}, respectively (Scheme 1). The K_d value obtained is close to those previously measured by isothermal titration calorimetry (0.1–4.6 μM)[39] while the values for k_{open} and k_{close} represent, to our knowledge, the first reported for human TG2.

These rate constants provide half-lives of 5.0 min for opening and 13.9 min for closing. From these values one can see that limited conformational interconversion takes place on the time scale of the CE separation (8–10 min elution time). It is also instructive to compare the k_{open} and k_{close} rate constants to k_{cat} values associated with the catalytic cycle. For the reaction of the highly homologous guinea pig liver TG2 with substrate analogues, k_{cat} values in the range of 17–114 min^{-1} have been reported.[33,40] Since the rate constants measured herein for the conformational changes associated with TG2 activation are much lower than k_{cat}, these conformational changes cannot be occurring during the catalytic cycle. This eliminates the possibility, for example, that the closed form reacts with a substrate, opens up upon acylation, and then closes up again upon the product release. Instead, it appears that a slow conformational change takes place upon activation of the enzyme, as a mechanism for functional regulation, and that once open, it then remains open throughout its catalytic cycle.

Finally, a simple kinetic experiment was performed to confirm the time scale of activation and deactivation. As shown in Figure S7 (Supporting Information), TG2 deactivation by calcium depletion was virtually instantaneous, consistent with rapid dissociation of calcium to an inactive calcium-unbound form. However, TG2 activation by calcium addition was slower, consistent with the rapid activation of existing open form TG2, followed by a slower shift in the conformational equilibrium from closed to open, on the minute time scale observed by KCE.

In summary, we have shown for the first time that KCE can be used to separate and detect the slowly interconverting open and closed conformations of human TG2. The addition of effector ligands affects this conformational equilibrium, in a manner consistent with previous structural data[15,17,30,31] and allowed the first direct measurement of the K_d value for calcium binding. These results provide important insight into the role of the slow conformational change in the functional regulation of TG2. The time scale of these conformational changes (seconds), compared to those associated with TG2 catalysis (milliseconds to microseconds) and those known for protein side-chain movement (nanoseconds to picoseconds) illustrate the complexity of the conformational landscape of TG2 and its relation to TG2 function.

We believe this study firmly establishes the broad utility of KCE as a powerful, complementary method for studying protein structure and function. While the slow conformational changes studied herein give rise to separate peaks on the electropherogram, faster conformational changes of other proteins could also be detected through peak broadening. Furthermore, this method could be extended to include detection of the in situ catalytic activity of separate conformers and their susceptibility to inhibitor binding.

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