High-throughput screening of holoprotein conformational stability by dynamic ligand exchange-affinity capillary electrophoresis†

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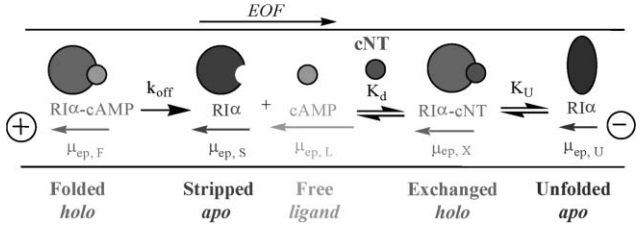
Dynamic ligand exchange-affinity capillary electrophoresis (DLE-ACE) is introduced as a convenient platform for assessing the conformational stability and relative affinity of a holoprotein to different ligands without off-line sample pretreatment, since ligand exchange and protein unfolding processes are integrated in-capillary during electromigration.

Ligand binding plays a crucial role in regulating protein conformation, activity and function. In order to assess the impact of a ligand on protein stability, conventional techniques based on optical spectroscopy, microcalorimetry and NMR compare the resistance to chemical or thermal denaturation of the ligand-free apoprotein relative to the holoprotein complex. However, off-line sample preparation of apoprotein and/or ligand-exchanged holoprotein by partial unfolding with equilibrium dialysis is time-consuming and labor-intensive.[1] Moreover, the *apo* state is often unstable and susceptible to proteolysis, whereas the preparation of a ligand-exchanged holoprotein can generate misfolding. Thus, new strategies are needed for the rapid preparation of different protein states when using small amounts of sample. This is the case for cyclic AMP (cAMP)-dependent protein kinase (cAPK), which is a ubiquitous holoprotein involved in cell signaling.[1] The inactive apoprotein tetramer consists of a regulatory dimer subunit and two catalytic subunits. cAMP binding to the regulatory subunits induces an allosteric conformational change, resulting in dissociation of catalytic subunits with kinase activity.[1] Herein, we demonstrate that DLE-ACE offers a single-step format for comparative *apo*/*holo* thermodynamic studies of a regulatory type I alpha (RIα) cAPK associated with different cyclic nucleotides (cNTs) as a model system. Since sample preparation is integrated during analysis, DLE-ACE is conducive for high-throughput screening of drug candidates for cyclic nucleotide binding proteins, such as guanine exchange factors and cGMP-dependent protein kinases.[2] Unlike recent high-throughput screening strategies based on MS,[3] DLE-ACE offers a unique format for *in-situ* generation and characterization of different protein states in solution without off-line apoprotein preparation and complicated sample handling procedures.

ACE is a high resolution microseparation technique that is increasingly being applied as a physical tool to quantify biomolecular interactions.[4] However, ACE is often not suitable for studying high affinity protein interactions with small ligands that induce negligible changes in protein mobility. Selective fluorescent labeling of the ligand (*e.g.* cAMP) can be performed to overcome this challenge; however, this can modify its intrinsic binding to a receptor, as well as imparting additional time and cost constraints on the assay.[5] Alternatively, ACE experiments can be performed *via* assessment of the relative stability of the *apo*/*holo* protein states upon unfolding.[3b,c] This strategy not only enables determination of apparent ligand dissociation constant (K_d), but also provides holoprotein conformational stability (ΔG°_U) and cooperativity (m) parameters. Although CE has been used to study protein unfolding, it is still an unrecognized technique for characterizing holoprotein systems.[6] Recently, our group demonstrated reversible *holo*-RIα-cAMP dissociation by CE, which permitted *in-situ* generation of the labile *apo* state *via* electrophoretic ligand-stripping.[6b] In this study, DLE-ACE with laser-induced native fluorescence (LINF) detection is introduced as a convenient yet rapid format for comparative thermodynamic studies of RIα involving different cNT analogues.



Scheme 1 Three-step process depicting dynamic cAMP-stripping, cNT exchange and *holo*-RIα-cNT unfolding by DLE-ACE, where arrow vectors represent the mobilities (μ_{ep}) of different protein/ligand states.

Scheme 1 illustrates the principle of DLE-ACE where the apoprotein (RIα) is first generated upon voltage application from the initial holoprotein (RIα-cAMP) that is injected as a short sample plug with excess cAMP. cAMP-stripping is mediated electrokinetically without chemical denaturant since the negative mobility of the small ligand is greater than the bulky ligand-stripped protein (*i.e.* $\mu_{ep,L} > \mu_{ep,S}$). This process is also favorable due to the fast dissociation rate constant ($k_{off} \approx 60\ s^{-1}$) for RIα[1d] when performing separations in buffer devoid of ligand. Owing to the reversibility of complex formation, ligand exchange can be realized during *apo*-RIα electromigration by the addition of excess cNT to the background electrolyte since the original ligand (*i.e.* cAMP) is irreversibly separated from the protein. When the same process is performed in the presence of urea, the exchanged *holo*-RIα-cNT (X) undergoes denaturation into the unfolded *apo*-RIα (U) state, where $\mu_{ep,U} < \mu_{ep,X}$. In this case, ligand-stripping and exchange must precede protein unfolding‡ (*i.e.* $k_{off} > k_U$) to ensure

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† The HTML version of this article has been enhanced with colour images.

adequate equilibration prior to detection, otherwise changes in buffer conditions will not alter the apparent thermodynamic parameters. Assuming a reversible two-state unfolding process for RIα,[1] the apparent free energy (ΔG_U) for unfolding can then be measured by changes in the viscosity-corrected apparent mobility (*i.e.* $v\mu_{ep}^{A}$) as a function of urea concentration.[6b] Alternatively, the fraction of unfolded protein (F_U) can be determined by eqn (1), which allows for direct comparison of protein denaturation curves:

$$F_U = \frac{v\mu_{ep}^{A} - \mu_{ep,F}}{\mu_{ep,U} - \mu_{ep,F}} \quad (1)$$

F_U data determined by DLE-ACE then allow determination of ΔG°_U and m by eqn (2) using the linear extrapolation method, where c is the urea concentration:

$$\Delta G_U = -RT\frac{F_U}{(1-F_U)} = \Delta G^{\circ}_U + mc \quad (2)$$

Comparison of ΔG°_U of *apo*/*holo* protein states associated with different cNT analogues also permits estimation of the apparent dissociation energy§ (ΔG°_d) of protein–ligand interactions,[1e]

$$\Delta G^{\circ}_d \approx \Delta\Delta G^{\circ}_U - RT\ln[L] \quad (3)$$

where, $\Delta\Delta G^{\circ}_U$ is the difference in free energy of the *apo*/*holo* states ($\Delta G^{\circ}_{U,holo} - \Delta G^{\circ}_{U,apo}$) and [L] is the free ligand concentration. The major advantage of DLE-ACE is that it permits direct injection of low amounts of stable holoprotein (<100 fmol per injection) without sample pretreatment, extrinsic fluorescent/radiolabels and sample pre-incubation. The latter feature was feasible due to fast unfolding kinetics of the protein relative to the separation timescale.

RIα is a weakly acidic (p$I \approx 5.3$)[6b] truncated protein construct (14.1 kDa) having two Trp residues at positions 188 and 222 within a single cAMP binding domain that serves as a good model for cyclic nucleotide binding.[7] Fig. 1(a) depicts an overlay of a series of electropherograms showing distinct mobility changes for *apo* and three different *holo*-RIα-cNT states in 6 M urea by DLE-ACE. All separations were performed using a short-end injection at the electrolyte outlet, which enabled rapid analyses of under 5 min. This faster separation format was also important for achieving more reliable mobility measurements of the labile *apo*-RIα state relative to our preliminary report,[6b] which was hampered by excessive peak broadening at low urea concentrations. This feature was critical for ensuring accurate relative holoprotein stability and affinity measurements. In fact, thermodynamic parameters measured for *apo*/*holo* states of RIα-cAMP in this study¶ were consistent with independent experiments performed by fluorescence spectroscopy on the wild-type RIα that contains two cAMP binding domains.[1c] It is apparent that each protein state (2) has a distinct stability reflective of its characteristic μ_{ep}^{A} relative to the neutral EOF marker (1) due to association with the cNT during electromigration. For instance, *apo*-RIα and *holo*-RIα-cGMP co-migrate as the unfolded protein state in 6 M urea, whereas *holo*-RIα-cAMP and *holo*-RIα-8BrcAMP migrate as partially unfolded and fully folded states, respectively. Thus, these studies qualitatively demonstrate that ligand binding has a differential impact on RIα stability that is associated with the magnitude of its affinity for each cNT analogue.

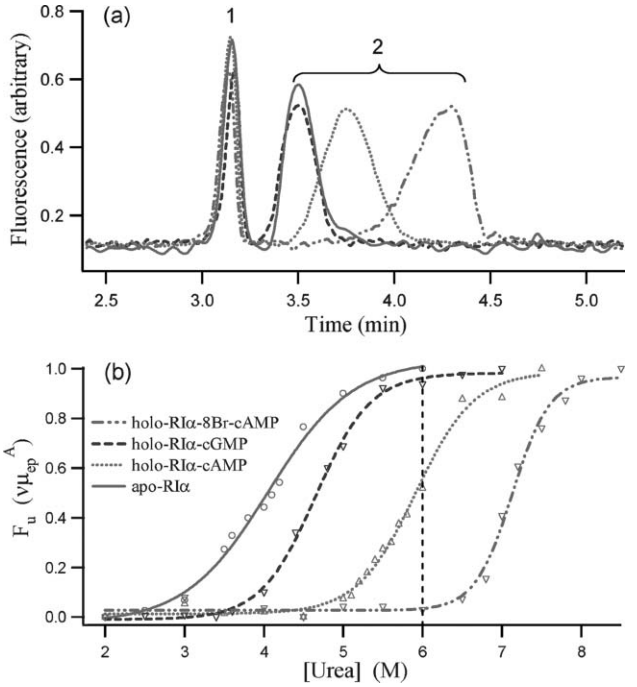

Fig. 1 Impact of ligand on conformational stability of RIα, where (a) depicts overlay electropherograms for different *apo*/*holo*-protein states in 6 M urea, and (b) compares *apo*/*holo*-protein unfolding curves as a function of cNT by DLE-ACE using LINF detection at 337 nm with 266 nm excitation.‖ Peaks correspond to (1) neutral EOF marker, melatonin and (2) RIα. Data are based on triplicate measurements with the coefficient of variation (CV) under 2%.

Fig. 1(b) compares the RIα unfolding curves as a function of excess cNT, representing four distinct protein states generated by DLE-ACE, namely (i) *apo*-RIα, (ii) *holo*-RIα-cAMP, (iii) *holo*-RIα-cGMP, and (iv) *holo*-RIα-8BrcAMP. It is clear that cAMP association results in enhanced stability of *apo*-RIα by about 6 kcal mol^{-1} with a 2 M increase in the urea mid-point concentration (C_M). In contrast, Fig. 1(b) demonstrates that excess cGMP confers a less significant increase in stabilization relative to *apo*-RIα of only 3 kcal mol^{-1}. Indeed, there is great interest in better understanding the cross-talk between cAMP and cGMP in the signal transduction pathway within cells since cAMP has been reported to activate cGMP-dependent protein kinases.[8a] However, binding of the membrane-permeable cAMP analogue, 8-BrcAMP, suggests a major enhancement in RIα conformational stability of 8 kcal mol^{-1} relative to *apo*-RIα, which is consistent with its higher affinity and full agonist activity.[8b] $\Delta\Delta G^{\circ}_U$ values in Table 1 can also be used to estimate dissociation constants using eqn (2). The relative affinity (*i.e.* $K_{d,cAMP}/K_{d,cNT}$) of 8-BrcAMP and cGMP to RIα was determined by DLE-ACE to be about 40-fold greater and 120-fold weaker than cAMP, respectively, which offers a more convenient format relative to traditional competitive radiolabel assays.[8b] In addition, it was determined that the unfolding cooperativity (m) for *holo*-RIα-cNT was similar for each cNT, but enhanced by about −0.7 kcal mol^{-1} M^{-1} relative to *apo*-RIα. This feature is useful for selecting ligand candidates in terms of their binding affinity and ordering effect on global protein conformation for subsequent structural or *in-vitro* activity studies.

Table 1 Comparison of thermodynamic parameters[a] for conformational stability and relative binding affinity of *holo*-RIα-cNT by DLE-ACE

holo-RIα (ligand)	ΔC_M[b]/M	Δm[b]/kcal mol^{-1} M^{-1}	$\Delta\Delta G^\circ_U$[b]/kcal mol^{-1}	$K_{d,cAMP}/K_{d,cNT}$
cAMP	1.9	−(0.7 ± 0.1)	(5.9 ± 0.5)	1
cGMP	0.7	−(0.6 ± 0.1)	(3.1 ± 0.5)	(0.083 ± 0.007)
8-BrcAMP	3.1	−(0.7 ± 0.1)	(8.1 ± 0.9)	(42 ± 5)

[a] Parameters derived from linear regression of RIα unfolding curves, where error represents ±1σ. [b] Differences in C_M, m and ΔG°_U of *holo*-RIα to *apo*-RIα states.

It is anticipated that DLE-ACE will play an increasing role as a promising platform for assessing the conformational stability of holoproteins with cyclic nucleotide binding domains. The advent of multiplexed capillary array instruments provides a high-throughput environment for screening ligand libraries for activity against protein targets. For instance, about 4500 ligands per day could be screened using a single commercial 96 capillary array electrophoresis instrument with a microtitre sample plate. This assumes a 10 min total run time per sample that includes capillary conditioning/sample handling, while using three urea denaturant conditions[3b] to estimate relative ligand affinity. Although the SUPREX method[3b] has been reported to be able to screen greater than 10 000 ligands per day, this does not take into account the time required for apoprotein preparation, which is often the major bottleneck in HTS of labile ligand-free receptor targets. To the best of our knowledge, no other technique offers similar advantages for seamlessly integrating sample preparation for the rapid characterization of holoprotein stability and the relative binding affinity to different ligands. Future work is directed at expanding the applicability of DLE-ACE for characterizing other protein–ligand systems. In conclusion, DLE-ACE offers a versatile format for comparative thermodynamic studies that is complementary with protein conformational mapping by NMR.[7a,b]

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Notes and references

‡ Scheme 1 is viable only if the rate of cAMP-stripping and cNT exchange is faster than protein unfolding [*i.e.* k_{off}(cAMP) > k_{on}(cNT) > k_U]. This feature ensures that irreversible separation of cAMP with cNT association occurs prior to urea-induced unfolding of *holo*-RIα-cNT in the background electrolyte. All protein samples were prepared in MES buffer in excess cAMP (0.2 mM) without urea in order to ensure long-term stability of the holoprotein. Thus, no sample pre-incubation in urea was required for equilibration prior to DLE-ACE, since RIα was observed to undergo fast unfolding kinetics relative to the separation timescale (*e.g.* 5 min). This property was verified by performing time-dependent unfolding studies of RIα (*i.e.* 0–1.5 h) pre-incubated in 4 and 6 M urea prior to CE without observing any significant changes in apparent protein mobility. The major advantage of DLE-ACE is that ligand-stripping/exchange processes can be readily controlled through simple modification of the electrolyte conditions.

§ This relationship assumes a reversible protein unfolding process where ligand interaction occurs only with the folded native protein state when using excess ligand that is equivalent to its equilibrium concentration (*i.e.* 200 μM). Refolding experiments were performed by unfolding *holo*-RIα-cAMP in 7 M urea off-line prior to DLE-ACE analyses in a buffer with excess cAMP but devoid of urea. No apparent misfolded or conformational intermediates were evident in these studies due to correlation in measured refolded protein $v\mu_{ep}^A$ with the folded state. This confirmed the reversibility of *holo*-RIα-cAMP unfolding and the validity of the measured thermodynamic parameters. All CE data were transformed into standard fraction of protein unfolding (F_U) plots in order to normalize the mobility values of the different protein states that facilitated comparative analyses.

¶ In this study, ΔG°_U for *apo*-RIα and *holo*-RIα-cAMP states were determined to be (3.7 ± 0.2) and (9.6 ± 0.5) kcal mol^{-1}, whereas m values were −(0.9 ± 0.1) and −(1.6 ± 0.1) kcal mol^{-1} M^{-1}, respectively. Improved correlation with literature values[1c] was achieved compared to our earlier study,[6b] notably for the labile *apo*-RIα by using a faster separation format to minimize protein band dispersion.

‖ All separations were performed on a P/ACE MDQ CE system equipped with dual-channel laser-induced fluorescence interface (Beckman-Coulter Inc., Fullerton, CA, USA). Uncoated fused-silica capillaries with 75 μm i.d., 360 μm o.d. and 30 cm length (Polymicro Technologies, Phoenix, USA) were used for the analyses. Each day the capillary was first conditioned by high pressure rinsing for 5 min with 0.1 M NaOH, 5 min in deionized water and 10 min in the background electrolyte. Subsequent unfolding studies were then preceded by a 2 min rinse with 0.1 M NaOH and a 3 min rinse with the background electrolyte prior to each separation. Owing to the high concentration of urea used throughout the experiments, the electrodes and capillary interface in the CE instrument were cleaned regularly each day to prevent significant salt residue accumulation and sample contamination. Separations were thermostated at 25 °C using a reversed polarity voltage of −8 kV since sample injections were performed at the electrolyte outlet. This short-end injection format enabled rapid analyses under 5 min with an effective capillary length of 10 cm to the detector. Although the capillary ends were not thermostated, this was not a significant issue since relative thermodynamic parameters were measured. In addition, separations were operated within the linear region of an Ohm plot to reduce electrolyte-induced heating effects. Native fluorescence excitation at 266 nm (average power output *ca.* 2 mW) was performed using a diode-pumped solid-state Q-switched Nd-YAG laser (CryLaS GmbH, Berlin, Germany) coupled to the CE instrument with a UV multimode fiber patchcord (Oz Optics, Carp, Ontario), whereas detection was performed at 337 nm using a narrow bandpass (FWHM ± 10 nm) emission filter (Andover Corporation, Salem, USA). Hydrodynamic injection of samples was performed at the capillary outlet using a low pressure (0.5 psi or 3.5 kPa) for 5 s. All separations were performed in 100 mM MES, pH 6.5 (adjusted by 0.1 M NaOH) buffer with excess cyclic nucleotide (200 μM cNT) and/or urea. Separation buffers containing cNT and urea were prepared prior to use from stock solutions in 100 mM MES pH 6.5. A stock solution of 10 mM melatonin was also prepared in water and added to the sample prior to analysis. DLE-ACE studies required less than 100 fmol of RIα (*e.g.* 40 μM in 30 μL) sample containing excess cAMP (280 μM). The concentration sensitivity can be further enhanced by using broad bandpass detection filters with appropriate digital noise filtering.

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