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Research paper

Differential scanning fluorimetry based assessments of the thermal and kinetic stability of peptide–MHC complexes

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ABSTRACT

Measurements of thermal stability by circular dichroism (CD) spectroscopy have been widely used to assess the binding of peptides to MHC proteins, particularly within the structural immunology community. Although thermal stability assays offer advantages over other approaches such as IC_{50} measurements, CD-based stability measurements are hindered by large sample requirements and low throughput. Here we demonstrate that an alternative approach based on differential scanning fluorimetry (DSF) yields results comparable to those based on CD for both class I and class II complexes. As they require much less sample, DSF-based measurements reduce demands on protein production strategies and are amenable for high throughput studies. DSF can thus not only replace CD as a means to assess peptide/MHC thermal stability, but can complement other peptide-MHC binding assays used in screening, epitope discovery, and vaccine design. Due to the physical process probed, DSF can also uncover complexities not observed with other techniques. Lastly, we show that DSF can also be used to assess peptide/MHC kinetic stability, allowing for a single experimental setup to probe both binding equilibria and kinetics.

1. Introduction

Quantifying the strength of the interactions between peptides and major histocompatibility complex (MHC) proteins is important in the identification of T cell epitopes and evaluating the consequences of naturally occurring or engineered peptide modifications. In many cases though, the instability of peptide-free "empty" MHC proteins (particularly for class I) has hindered direct measurements of MHC–peptide binding equilibria. To circumvent this complexity, a variety of experimental approaches have been developed that yield proxies for MHC–peptide binding affinities. Perhaps the most common is the use of competition experiments to report IC_{50} values relative to a labeled competitor (Ruppert et al., 1993). Other assays have been developed, including the use of pre-oxidized protein to reduce instability issues (Harndahl et al., 2009) and indirect assays that rely on antibody or T cell receptor detection of peptide/MHC complexes (Miles et al., 2011).

In addition to the approaches above, measurements of peptide/MHC thermal stability are commonly used to assess how well peptides interact with MHC proteins. Folded proteins exist in equilibrium with their unfolded states, and due to the principle of coupled equilibria, ligand binding to a folded protein will increase protein thermodynamic stability. Due to this link, the presence of a bound ligand increases protein thermal stability, and the increase in a protein's T_m (formally defined as the temperature at which 50% of the protein is unfolded) correlates with binding affinity (Crothers, 1971; Brandts and Lin, 1990). Measurements of peptide/MHC thermal stability have been widely adopted by the structural immunology community, with circular dichroism (CD) spectroscopy being the most frequent mode of detection. In this use, CD monitors the loss of α-helical character in the peptide binding domain as the complex dissociates and unfolds, and early studies demonstrated close agreement between peptide/MHC thermal stability and robust affinity determinations by equilibrium dialysis (Morgan et al., 1997). Thermal stability measurements, whether by CD or other approaches (Schlundt et al., 2009; Schlundt et al., 2012) are advantageous in that they are not dependent on the identity or concentration of a competitor peptide. They can also be easily implemented, as they do not require peptide or protein modifications and use relatively simple instrumentation.

A drawback of CD-monitored thermal unfolding of peptide/MHC complexes is the limited signal-to-noise ratio and the large amount of sample required (typically hundreds of microliters of protein at concentrations of tens of micromolar). Although small relative to the significant amount of protein typically needed for structural studies, these

Abbreviations: DSF, differential scanning fluorimetry; T_m, melting temperature; CD, circular dichroism.

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requirements nevertheless reduce throughput and limit the use of thermal stability measurements in broader studies such as epitope discovery. Although small volume CD systems can reduce sample requirements, these suffer from low signal-to-noise, and medium-throughput CD instrumentation requiring complex liquid handling robotics has only recently been described (Fiedler et al., 2012).

Differential scanning fluorimetry (DSF; also referred to as ThermoFluor) provides an alternative to CD spectroscopy as a method to assess protein stability and the impact of ligand binding (Pantoliano et al., 2001; Matulis et al., 2005; Niesen et al., 2007). DSF takes advantage of small, environmentally-sensitive fluorescent molecules whose fluorescence is enhanced when specifically bound to exposed hydrophobic surfaces such as those created by protein unfolding. Due to the high sensitivity of modern fluorimeters, DSF is well suited to low volume, rapid measurements on multiple samples. DSF has found numerous uses in characterizing protein stability and ligand binding, and has been recently used to assess the binding of peptides to class I MHC proteins (Gras et al., 2012; Koch et al., 2013a; Koch et al., 2013b; Hassan et al., 2015), as well as the impact of peptides and other molecules binding to class II proteins (Günther et al., 2010; Rupp et al., 2011; Clayton et al., 2014), including HLA-DM (Álvaro-Benito et al., 2015). Although early applications of DSF required customized fluorimeters, the technique is easily adaptable to fluorescence-equipped RT-PCR instruments common to many molecular biology laboratories. DSF is thus highly scalable, with one experimental run capable of assaying dozens of small-volume samples in plate format. Here we performed a comparison of DSF and CD spectroscopy for measuring the thermal stability of both class I and class II peptide/MHC complexes. We found DSF a straightforward, easily implementable approach that yields results equivalent to those obtained from CD spectroscopy while considerably reducing sample requirements.

Lastly, interest in peptide/MHC kinetic as opposed to thermodynamic stability has been growing. In some instances, kinetic stability may be a more biologically relevant parameter, as it more accurately reflects the situation of a MHC-presented peptide on a cell surface. Indeed, some reports indicate that kinetic stability is an improved indicator of antigenicity and immunodominance than binding affinity (Lazarski et al., 2005; Baumgartner et al., 2010; Harndahl et al., 2012). Various approaches have been used to measure peptide–MHC kinetic stability, including using fluorescent or radioactive peptides, surface plasmon resonance, and a recently developed scintillation proximity assay (Gakamsky et al., 1996; Gakamsky et al., 2000; Binz et al., 2003; Baxter et al., 2004; Harndahl et al., 2011; Miles et al., 2011). We show here that for class I peptide/MHC complexes, DSF readily lends itself to direct measurements of peptide dissociation without the need for protein or peptide labeling.

2. Materials and methods

2.1. Peptides and proteins

Peptides were synthesized commercially (CHI Scientific or 21st Century Biochemicals). For class I protein, recombinant HLA-A*0201 heavy chain and β_2-microglobulin were expressed as inclusion bodies in *Escherichia coli* (Garboczi et al., 1992). MHC folding and assembly from inclusion bodies was performed according to standard procedures (Pierce et al., 2014) Protein was purified using ion exchange followed by size-exclusion chromatography. For class II protein, soluble extracellular domains of HLA-DR1 (DRA*0101/DRB*010101) were expressed in *Drosophila* S2 cells and purified by immunoaffinity chromatography as previously described (Sloan et al., 1995).

2.2. Differential scanning fluorimetry

Differential scanning fluorimetry was performed using Applied Biosystems StepOnePlus (for class I complexes) or Bio-Rad C1000 Thermal Cycler (for class II complexes) RT-PCR instruments with the excitation and emission wavelengths set to 587 and 607 nm, respectively. For class I complexes, solution volumes were 20 μL in 96-well plates. Assay buffer was 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, with 0.005% surfactant P20 added to prevent protein adherence to the plate. For class II complexes, solution volumes were 25 μL in 96-well plates. Assay buffer was 100 mM sodium citrate (pH 5.5), 50 mM NaCl, 5 mM EDTA, 0.1% NaN3, 1 mM DTT, 1 mM PMSF, and 0.1% octylglucoside. The different solution conditions for class I and class II complexes were based on optimal conditions for preparation and storage of the different complexes, and are conventional buffers for the two systems (Khan et al., 2000; Yin et al., 2015). For class II complexes, the lower pH is believed to approximate conditions for endosomal loading. Pilot studies of class I complexes under the conditions used to assess class II resulted in substantial reductions in protein stability.

For thermal stability measurements, the temperature scan rate was fixed at 1 °C/min unless otherwise indicated. Protein and SYPRO orange (Invitrogen) concentrations were varied as described. The temperature range spanned 20 °C to 95 °C. Data analysis was performed in OriginPro 7 or 9. Apparent T_m values were first determined by identifying the point at which the transition was 50% complete. For a more rigorous analysis, the temperature derivative of the melting curve was computed. The resulting derivative curve was processed with the peak fitting algorithm in OriginPro, applying a sigmoidal baseline and fitting the peak to determine the T_m and its standard error. A bi-Gaussian function, commonly used in spectroscopy and chromatography, was used for peak fitting as the peaks were usually noticeably skewed, presumably due to contributions from irreversible aggregation that occurs coincident with unfolding. When multiple transitions were observed, the data were subject to the automated multiple peak fitting process in OriginPro, using default options except that bi-Gaussian functions were used. This proved successful in identifying the major transitions and their associated T_m and standard error as shown in Figs. 1C and 3A. For kinetic measurements, 10 μM of protein was incubated in the instrument at a constant temperature of 37 °C with 10× SYPRO orange, with fluorescence measured every 5 min. Kinetic data were fit to a biexponential function. As performed previously (Binz et al., 2003), the slowest rate constant was attributed to peptide dissociation from the class I MHC heterotrimer and used to determine half-lives.

2.3. Circular dichroism spectroscopy

CD spectroscopy was performed as previously described (Khan et al., 2000; Borbulevych et al., 2010; Borbulevych et al., 2011; Ekeruche-Makinde et al., 2012), using a Jasco J815 instrument. Temperature was increased from 10 °C to 100 °C at an increment of 1 °C/min, monitoring a wavelength of 218 nm. Protein concentrations were between 5 μM and 10 μM in 20 mM phosphate (pH 7.4), 75 mM NaCl. Data analysis was performed in Kaleidagraph or OriginPro. Data in the transition region were differentiated or fit to a polynomial, and apparent T_m values were determined as the point in which the transition was 50% complete.

2.4. IC_{50} measurements

IC_{50} measurements for peptide binding to HLA-DR1 were determined using a fluorescence polarization assay, using Alexa488-labeled $HA_{306–318}$ as the indicator peptide as previously described (Yin et al., 2012; Yin and Stern, 2014).

3. Results

3.1. Implementation and optimization of DSF for peptide/MHC stability

Differential scanning fluorimetry was implemented utilizing the environmentally-sensitive fluorescent molecule SYPRO orange. SYPRO