FOR THE RECORD

Circular dichroism determination of class I MHC–peptide equilibrium dissociation constants

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Abstract: Class I major histocompatibility complex (MHC) molecules bind peptides derived from degraded proteins for display to T cells of the immune system. Peptides bind to MHC proteins with varying affinities, depending upon their sequence and length. We demonstrate that the thermal stability of the MHC–peptide complex depends directly on peptide binding affinity. We use this correlation to develop a convenient method to determine peptide dissociation constants by measuring MHC–peptide complex stability using thermal denaturation profiles monitored by circular dichroism.

Keywords: circular dichroism; class I MHC; dissociation constant

Class I major histocompatibility complex (MHC) proteins bind fragments of cytosolic proteins for presentation to the immune system. These cell-surface proteins are heterodimers, composed of a heavy chain (~45 kD), a noncovalently associated light chain, β_2 -microglobulin (β_2m , 12 kD), and a noncovalently bound peptide (typically 8–10 residues). The heavy chain is composed of three domains, α_1 , α_2 , and α_3 , and contains a peptide binding groove formed by two α -helices and a β -sheet of domains α_1 and α_2 (reviewed in Bjorkman & Parham, 1990).

MHC proteins derived from different alleles have preferences for particular amino acids, called anchor residues, at specific positions in the peptide sequences. For example, the murine class I MHC protein H-2K^d has a preference for binding peptides containing tyrosine at position two (P2), and a hydrophobic amino acid at position nine (P9), as in the peptide NP1 (TYQRTRALV, see Table 1). The dissociation constant for the K^d/NP1 complex is two orders of magnitude lower than the complex formed with NP10, an analogous peptide with phenylalanine at P2. A peptide

variant with threonine at P2 has no detectable binding to K^d. Similarly, NP1 binds to K^d three orders of magnitude more tightly than NP15, a peptide with the P9 hydrophobic residue valine of NP1 replaced by serine (Fahnestock et al., 1994). Therefore, in K^d, as in other MHC molecules, the identities of the anchor residue are responsible for the majority of the binding specificity.

Empty class I molecules lacking peptides are considerably less stable than their filled counterparts (Ljunggren et al., 1990; Schumacher et al., 1990; Townsend et al., 1990). Circular dichroism (CD) thermal melting curves of empty and peptide-filled K^d were used to demonstrate that the binding of a peptide confers about 4.4 kcal/mol of stability to the empty heterodimer (Fahnestock et al., 1992). Different peptides stabilize MHC molecules by different amounts, as evidenced by the dissociation rate of β_2m , which is

Table 1. Sequences, thermal denaturation temperatures, and dissociation constants for nonameric peptides complexed to K^d

Peptide	Sequence	T_m (°C)	K_D (M) ^a	K_D (M) ^b
			determined by equilibrium dialysis	determined by CD thermal denaturation
KD1	SYFPEITHI	61.0	4.7×10^{-8}	6.6×10^{-8}
NP1 ^c	TYQRTRALV	61.0	6.9×10^{-8}	6.6×10^{-8}
NP22	TYQRTCALV	58.0	9.5×10^{-7}	1.1×10^{-6}
NP10	TFQRTRALV	57.0	5.6×10^{-6}	2.8×10^{-6}
NP15	TYQRTRALS	55.0	3.0×10^{-5}	1.8×10^{-5}
NP17	TYQRTRALK	52.0	2.7×10^{-4}	3.0×10^{-4}
NP25	TYGGGGGLV	52.0	1.5×10^{-4}	3.0×10^{-4}
No added peptide ^d	—	45.0	—	—

^aFahnestock et al., 1994.

^bThis study.

^cThe previously reported T_m for the NP1–K^d complex is 57°C (Fahnestock et al., 1992). In this study, a higher T_m is observed for the same complex, possibly because the protein used in the previous study was denatured and reassembled prior to the addition of peptide.

^dApproximately 30% of this protein contains a mixture of endogenous peptides, as discussed in the text.

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reflective of MHC-peptide complex stability (Parker et al., 1992). However, the amount of stability the peptide confers to the MHC-peptide complex and how tightly the peptide binds have not been correlated by this method. In the case of the murine class I MHC molecule H-2K^d, peptide equilibrium dissociation constants have been measured by equilibrium dialysis assays (Fahnestock et al., 1994). This paper establishes a correlation between the thermal denaturation temperatures and the dissociation constants of K^d-peptide complexes.

Results and discussion: A soluble version of the murine class I MHC protein, H-2K^d, was loaded with peptides by incubation with a 10-fold molar excess of peptide at room temperature for 12 h. Approximately 30% of the K^d molecules as purified from transfected cell supernatants were occupied with endogenous peptides (Fahnestock et al., 1992). This initial semi-filled population is expected to make only a small contribution to the apparent melting temperature (T_m).

Thermal denaturation curves of K^d-peptide complexes were measured by monitoring the CD ellipticity at 223 nm (Fig. 1). Melting temperatures were determined for the K^d-peptide complexes listed in Table 1. The dissociation constants (K_D s) of these peptides, which range over four orders of magnitude, were previously measured by equilibrium dialysis (Fahnestock et al., 1994). Despite the presence of endogenous peptides in a fraction of the MHC proteins, we found a logarithmic relationship between the T_m s of the K^d-peptide complexes and the peptide dissociation constants for nonameric peptides (Fig. 2). These results demonstrate that the amount of thermal stability the peptide imparts to the K^d-peptide complex is a direct result of how tightly the peptide binds.

The equation relating K_D and T_m for those peptides with known dissociation constants can be used to calculate dissociation constants for nonameric peptides (Table 1). The error in the T_m mea-

Fig. 1. Representative thermal denaturation curves of K^d without exogenous peptide (squares), complexed to NP25 (triangles), and complexed to NP22 (circles). The curves have been smoothed and normalized.

Fig. 2. K_D versus T_m for the peptides listed in Table 1. The equation relating K_D and T_m is $K_D = 10^{[(43.3 - T_m)/2.47]}$, $R = 0.990$.

surement is estimated to be $\pm 0.5^\circ\text{C}$, which results in an error in the calculated K_D of approximately half an order of magnitude, which is comparable to the error in equilibrium dialysis determination of K_D .

Given a set of peptides with known dissociation constants that can be used to generate a standard curve, it should be possible to determine K_D s by thermal denaturation for other class I MHC proteins. This methodology may extend to class II MHC proteins and other proteins that bind peptides, such as the viral MHC homolog UL18 (Fahnestock et al., 1995).

One issue with this methodology concerns the octameric peptide KD3, YIPSAEKI. Its T_m was determined to be 57°C , which results in a calculated K_D that is significantly larger than the K_D measured by equilibrium dialysis. Although the crystal structure of an octamer bound to MHC Class I molecule H2-K^b reveals the octamer in an extended conformation (Fremont et al., 1992), this may not be the case for KD3 bound to K^d. T_m measurements are comparable only for complexes with peptides bound with the same backbone conformation. The first residue of KD3 is tyrosine, which presumably binds as an anchor residue in the P2 pocket, which would result in the loss of all N-terminal contacts. The loss of N-terminal contacts between the peptide and MHC protein has been previously shown to destabilize an HLA-A2 MHC-peptide complex by 4.6 kcal/mol (Bouvier et al., 1994). This may explain why KD3 has lower thermal stability than expected judging from its binding constant.

Materials and methods: *Expression and purification of soluble H2-K^d in CHO cells:* A stable CHO cell line expressing a secreted form of K^d was used as previously described (Fahnestock et al., 1992). The protein was purified using immunoaffinity columns constructed with immobilized monoclonal antibodies, either 34-1-2 (anti-H2-K^d) (Ozato et al., 1982) or BBM.1 (anti- $\beta_2\text{m}$) (Parham et al., 1983). Approximately 70% of the purified protein product does not contain endogenous peptides (Fahnestock et al., 1992).

Peptide synthesis: Peptides were synthesized on an Applied Biosystems 433A peptide synthesizer. Preloaded resins were used, with subsequent residues coupled via Fmoc chemistry and HTBU/HOBt activation with standard 0.25 mmol scale coupling cycles. Peptides were cleaved from the solid support resin by mixing 200 mg resin with 2 mL trifluoroacetic acid (TFA), 100 μ L water, 150 mg phenol, 100 μ L thioanisole, and 50 μ L ethanedithiol for 2 h. The peptides were precipitated by addition of cold methyl *tert*-butyl ether, washed four times with the same solvent, and lyophilized to partially remove the cleavage reaction scavengers. The peptides were further purified by reverse-phase HPLC with a Vydac C8 column using linear acetonitrile-water gradients (typically 20–30%) containing 0.1% TFA. Peptide masses were determined by MALDI-TOF mass spectrometry and were found to be within one mass unit of the expected masses.

CD thermal denaturation curves: An Aviv 62A DS spectropolarimeter equipped with a thermoelectric cell holder was used to collect CD data. Data were obtained from samples containing 10 μ M MHC and 100 μ M peptide in 5.0 mM sodium phosphate, pH 7.0 using a 1.0 mm path length cell. Thermal denaturation curves were recorded at 223 nm over an appropriate temperature range with a 0.1 s time constant, 10 s averaging time, 3 min equilibration time, and 1 nm bandwidth. Melting temperatures were determined by taking the maximum of a plot of $d(\text{ellipticity})/dT$ versus T after averaging the data with a moving window of 3°. The error in T_m is estimated to be $\pm 0.5^\circ\text{C}$.

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