Molecular Immunology 54 (2013) 386–396

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Not all empty MHC class I molecules are molten globules: Tryptophan fluorescence reveals a two-step mechanism of thermal denaturation

Sunil Kumar Saini, Esam Tolba Abualrous, Anca-Sarmiza Tigan, Kathryn Covella, Ursula Wellbrock, Sebastian Springer*

Molecular Life Science, Jacobs University Bremen, Bremen, Germany

ARTICLE INFO

Article history:
Received 20 September 2012
Received in revised form
26 December 2012
Accepted 4 January 2013
Available online 4 February 2013

Keywords:
Tryptophan fluorescence
Thermal denaturation
Major histocompatibility complex class I molecules
Ligand binding kinetics
Protein folding
Protein glycosylation

ABSTRACT

When major histocompatibility complex (MHC) class I molecules bind peptide, they change their conformation and their dynamics. The structure and properties of the peptide-empty class I are still largely unknown. We have investigated the thermal denaturation of the murine class I allotypes H-2D^b and H-2K^b through the fluorescence of their intrinsic tryptophans, and we find that it occurs *via* an empty form that can also be produced by folding denatured recombinant class I molecules. It rapidly binds exogenous peptides. Our data demonstrate that the empty form of class I is a distinct conformational state with at least transient stability.

1. Introduction

MHC[1] (major histocompatibility complex) class I molecules are central to the cellular immune response. They are transmembrane peptide receptor proteins that bind peptides in the lumen of intracellular compartments and then travel to the cell surface to present them to cytotoxic T lymphocytes. Peptide selection by class I molecules is central to immunodominance, since only peptides that are loaded efficiently under cellular conditions are used in the immune response (van der Burg et al., 1996; Yewdell, 2006). After synthesis and folding in the endoplasmic reticulum (ER), class I molecules bind to peptide cohorts of progressively higher affinity in a process termed peptide optimization (Williams et al., 2002), which is assisted by the chaperone protein tapasin (Chen and Bouvier, 2007; Praveen et al., 2010; Wearsch and Cresswell, 2007). High-affinity peptides fit well into the binding groove of class I because of their length (8–10 amino acids) and anchor residue side chains at defined positions (Madden, 1995). The molecular details of the peptide binding and exchange processes are still unclear. Complex kinetic data suggest a mechanism with several steps (Gakamsky et al., 1999; Springer et al., 1998).

One central impediment to the mechanistic study of peptide binding to class I is that very little is known about empty class I molecules. Since bacterially expressed class I molecules refolded without peptide have not yielded crystal structures so far, the peptide has often been called the "third subunit" of class I, which implies that an empty binding site would exist only in an unfolded or "molten globule" state (Bouvier and Wiley, 1998; Silver et al., 1991). In contrast to this assertion, peptide-receptive class I molecules (defined as class I molecules that can rapidly bind exogenous peptide) have been demonstrated in detergent lysates and in permeabilized cells (Day et al., 1995; Townsend et al., 1989). Such class I molecules have also been isolated in a pre-folded and glycosylated form from mammalian cells and purified by size exclusion chromatography, and they were able to bind exogenous peptides on a time scale of seconds in *in vitro* experiments, demonstrating that they did not contain high-affinity ligand (Bouvier and Wiley, 1998; Fahnestock et al., 1994; Springer et al., 1998). Such peptide-receptive class I molecules may be not entirely "empty" since they might still contain low-affinity (*e.g.* truncated) peptides or nonpeptidic ligands such as glycerol. Thus, even in the absence of crystals,

* Corresponding author at: Molecular Life Science, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany. Tel.: +49 421 200 3243; fax: +49 421 200 3249.
E-mail address: sebastian.springer@queens.oxon.org (S. Springer).

[1] Abbreviations: β_2m, beta-2 microglobulin (the light chain of MHC class I); CD, circular dichroism; DSC, differential scanning calorimetry; ER, endoplasmic reticulum; MHC, major histocompatibility complex; TDTF, thermal denaturation measured by tryptophan fluorescence; T_m, midpoint of thermal denaturation transition ("melting point"). All peptides are abbreviated in the single letter amino acid code.

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http://dx.doi.org/10.1016/j.molimm.2013.01.004

some evidence exists that class I molecules are stable, at least for some time, without a high-affinity ligand.

In this work, we have studied the thermal stability of class I-peptide complexes using the fluorescence of their endogenous tryptophans. We demonstrate that their denaturation proceeds *via* an empty but folded peptide-receptive state of class I, and we find that such class I molecules can be obtained by *in vitro* folding reactions without peptide.

2. Results

2.1. Tryptophan fluorescence faithfully monitors class I stability

We first produced the extracellular domains of the murine class I allotypes, H-2D^b and H-2K^b and the human light chain beta-2 microglobulin (hβ_2m) in *Escherichia coli* as insoluble inclusion bodies, denatured the proteins, and folded heavy chain–hβ_2m–peptide complexes *in vitro* according to published protocols, with the respective peptide present in the folding reaction (Garboczi et al., 1992).

To measure the thermostability of the class I-peptide complexes, we used TDTF (thermal denaturation measured by tryptophan fluorescence), a label-free real-time method that uses small amounts of protein (Eftink, 1994). TDTF is measured in a fluorimeter cuvette that is slowly heated with a Peltier heater and monitored by an in-cuvette thermocouple. The intensity of the fluorescence of the endogenous tryptophan side chains of the protein (λ_{ex} = 290 nm, λ_{em} = 345 nm) is measured, which depends on the dielectric constant of their environment in the protein (Lakowicz, 2006). When the protein unfolds, the tryptophan side chains are exposed to the aqueous environment of a lower dielectric constant, and the fluorescence intensity decreases. Since protein unfolding is usually a cooperative process, the tryptophan signal usually decreases in a single distinct step; we also observed this with our class I-peptide complexes, such as the complex of H-2D^b with the Sendai virus nucleoprotein 324–332 peptide, FAPGNYPAL (single letter amino acid code; Fig. 1A top panel). The inflection point of the curve (arrow) is called the midpoint of transition (T_m), or "melting point." It is easier to visually estimate as the minimum of the first derivative of the fluorescence curve (48.2 ± 0.19 °C; Fig. 1A, bottom panel). We and others have shown previously that the T_m of a class I-peptide complex measured by TDTF relates to the binding affinity of the ligand peptide (Glithero et al., 2006; Hulsmeyer et al., 2005; Pohlmann et al., 2004; Springer et al., 1998).

We wished to compare TDTF to differential scanning calorimetry (DSC), where unfolding is measured *via* the heat energy required to break the secondary and tertiary structure of the protein. The T_m of the H-2D^b-FAPGNYPAL complex measured by DSC (51.2 ± 0.06 °C; Fig. 1B and Table 1) was slightly higher than that from TDTF, suggesting that the higher protein concentrations in the DSC measurement (1 mg/ml, *vs.* 40 μg/ml in TDTF) had stabilized the protein. Over many (>20) repeats, the values derived from TDTF were reproducible.

To compare TDTF with circular dichroism (CD), which measures the loss of secondary structure upon unfolding of the protein, we prepared complexes of another murine allotype, H-2K^b, hβ_2m, and the lymphocytic choriomeningitis virus glycoprotein 34–41 peptide AVYNFATM and measured the T_m by TDTF. We found that our melting point of 61.0 ± 0.03 °C (Fig. 1C) was close to the published CD value, 56.7 ± 0.1 °C (Madhurantakam et al., 2012). A similar good agreement between TDTF and CD was found elsewhere (Pohlmann et al., 2004). Taken together, the results of these experiments suggest that TDTF is a valid technique for determining the thermal stability of class I-peptide complexes.

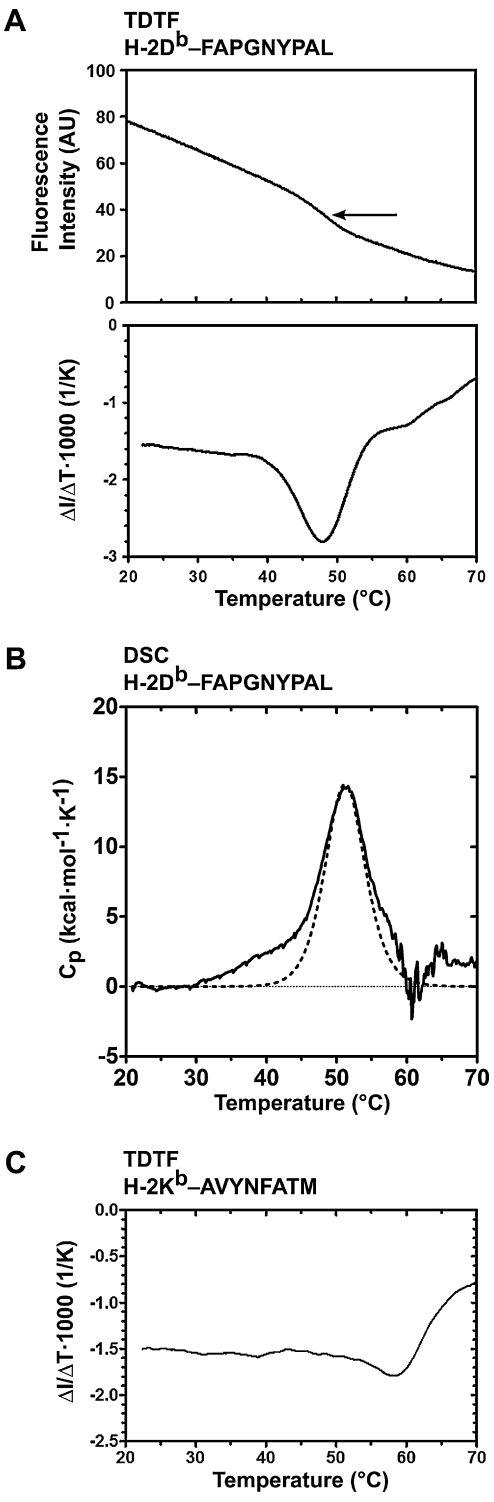

Fig. 1. Thermal denaturation measured by tryptophan fluorescence (TDTF) yields T_m values compatible with established techniques. Representative experiments are shown are shown along with averaged T_m values and their SEM where applicable. A summary of the data with the number of repeats of each experiment is shown in Table 1. A, TDTF measurement of 40 μg/ml H-2D^b-hβ_2m in complex with the FAPGNYPAL peptide. The fluorescence intensity curve shows a decrease of fluorescence with temperature and a drop upon unfolding of the protein; the transition midpoint is the T_m (arrow; upper panel). The LOWESS fit to the first derivative shows the T_m as a minimum at 48 °C (lower panel). B, differential scanning calorimetry (DSC). H–2D^b-hβ_2m was folded with the peptide FAPGNYPAL and, at a protein concentration of 1 mg/ml, subjected to DSC. The dashed line shows a theoretical fit with T_m = 51.3 ± 0.06 °C. Heating rates were 0.3 K/min in both experiments, but the protein concentration was 25-fold higher in the DSC experiment. C, TDTF of H-2K^b-hβ_2m with AVYNFATM yields a T_m of 58 °C.