Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, refolding, crystallization and preliminary crystallographic study of MHC H-2K^k complexed with octapeptides and nonapeptides

Christine Kellenberger,[a]* Sophie Porciero[b] and Alain Roussel[b]

[a]Centre d'Immunologie de Marseille-Luminy, UMR 6102, Parc Scientifique et Technologique de Luminy – Case 906, 13288 Marseille, France, and [b]AFMB UMR 6098 CNRS, 31 Chemin J. Aiguier, 13402 Marseille CEDEX 20, France

Correspondence e-mail: kellenberger@ciml.univ-mrs.fr

Received 24 March 2004
Accepted 19 April 2004

Major histocompatibility complex (MHC) molecules are heterodimeric cell-surface receptors that play a crucial role in the cellular immune response by presenting epitope peptides to T-cell antigen receptors (TCR). Although the structural basis of the peptide–MHC binding mechanism is becoming better understood, it is still difficult to predict a binding mode for an MHC of unknown structure. Therefore, as the first stage of a TCR–MHC interaction study, the crystal structures of the mouse H-2K^k molecule in complex with both an octapeptide from *Influenza A virus* and a nonapeptide from simian virus SV40 were solved. Here, the expression, refolding, purification and crystallization of the two complexes are reported. For the H-2K^k–HA(259–266) complex, crystals were obtained *via* an extensive screen using a nanodrop-dispensing robot and diffracted to 2.5 Å resolution. For the H-2K^k–SV40(560–568) complex, microscopic needles were initially obtained and their size was improved by macroseeding and a stepwise increase in precipitant concentration. Diffraction data to a resolution of 3.0 Å were collected at a synchrotron facility.

1. Introduction

The recognition of MHC-bound peptides by TCR lies at the centre of the adaptative immune system (Townsend & Bodmer, 1989). The MHC class I family comprises a large number of different molecules characterized by a high degree of polymorphism, each specific molecule binding a large set of peptides (typically 8–10 amino acids). During the last 10 y, a large number of structural studies of MHC class I molecules have been reported. In particular, H-2K^b (PDB code 2vaa; Fremont *et al.*, 1992) and HLA-A2 (PDB code 1hhg; Madden *et al.*, 1993) have been extensively studied in complex with various peptides. This work has provided fundamental insights into the mode of the peptide–MHC binding mechanism. In particular, interactions are achieved between the backbone of the antigenic peptide and the side chains of conserved MHC residues in the binding groove. The polymorphic amino acids define the A–F pockets of the antigen-binding cleft (Matsumura *et al.*, 1992). These pockets complement a small number of specific amino acids; thus, the peptides that bind to a specific MHC class I allele have in common two or three 'anchor' residues that are found to be relatively invariant. These anchors interact through their side chains with MHC specificity pockets. At present, a large number of structural studies are available for human HLA-A and HLA-B (for example, HLA-B35; PDB code 1a1n; Smith *et al.*, 1996) molecules, while for mouse MHC, in addition to H-2K^b, structural studies have been reported on H-2L^d (PDB code 1ldp; Speir *et al.*, 1998), H-2D^b (PDB code 1n5a; Achour *et al.*, 2002) and H-2D^d (PDB code 1bii; Achour *et al.*, 1998). In the present study, we focus on the H-2K^k allele (Pullen *et al.*, 1992), which presents 90% identity to the H-2K^b molecule but displays different anchor residues. H-2K^k selects peptides with a Glu at P2 and an Ile/Val at the PΩ position, while the pocket architecture of H-2K^b results in a preference for aromatic residues at P5 and Leu/Val/Ile/Met at the PΩ position. As a first stage in a TCR–MHC interaction study, we have undertaken the solution of the structure of H-2K^k in complex with an octapeptide HA(259–266) from *Influenza A virus* (Gould *et al.*, 1991) and with a nonapeptide SV40(560–568) from simian virus (Rawle *et al.*, 1988) in order to define its proper mode of binding. Here, we report the expression, refolding and purification of the two complexes. We also describe the processes of crystallization, which differ for the two complexes.

2. Experimental and results

2.1. Expression, refolding and purification

Truncated H-2K^k heavy chain (279 residues) and β-2 microglobulin (β2m; 99 residues) cloned in pET3a plasmid were overexpressed in *Escherichia coli* BL21-pLysS bacteria as inclusion bodies. These inclusion bodies were

washed in a lysis buffer using 0.5% Triton X-100, solubilized in 8 *M* urea and subjected to refolding in the presence of either SV40(560–568) or HA(259–266). Two methods were evaluated for the production of ternary complexes: dilution and dialysis. In the first instance, the dilution method was assayed with the nonameric peptide SV40(560–568). The inclusion bodies (heavy chain:β-2m ratio 1:3) and the peptide (heavy chain:peptide ratio 1:10) dissolved in 8 *M* urea were rapidly diluted (~250–500-fold) in the refolding buffer to a final concentration of 30–50 mg l^{-1} protein. Varying concentrations of Tris, NaCl, Arg, EDTA and urea in the presence of glutathione (GSH/GSSG, 5/0.5 m*M*) were tested. This method, in the presence of 100 m*M* Tris, 400 m*M* Arg and 2 m*M* EDTA, yielded the properly refolded complex in low yield and a significant amount of a side-product. The molecular weight of this side-product was determined by MALDI–TOF mass spectrometry. It corresponds to a complex of the modified heavy chain (theoretical MW + 311 Da), β-2 microglobulin and the peptide.

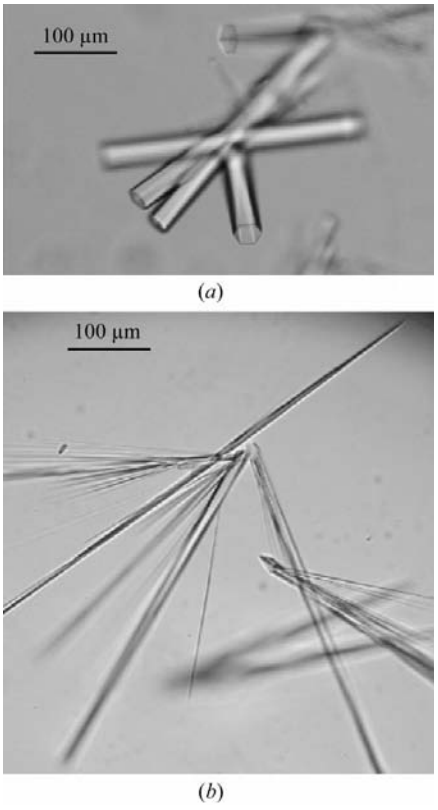

Figure 1
Crystals of MHC H-2K^k complexed with octapeptides and nonapeptides. (*a*) Crystals of H-2K^k in complex with the octapeptide HA(259–266) from influenza A virus appear in few days and diffract to 2.5 Å resolution. (*b*) Crystals of H-2K^k in complex with the nonapeptide SV40(560–568) are needles of about 10 µm width. The diffraction limit is 3.0 Å resolution using synchrotron radiation.

A second approach based on dialysis was also assayed. The solubilized inclusion bodies were mixed with the peptide (heavy chain:β-2m:peptide ratio 1:3:10) to a final protein concentration of 0.5 mg ml^{-1} in 6 *M* urea, 1 m*M* EDTA and 10 m*M* Tris pH 8.0. A stepwise dialysis was performed against 2 *M* urea, 1 *M* urea and 0 *M* urea in the same buffer solution. This method also resulted in low yields, but no side-product was detected and it was more straightforward and was thus preferred over the dilution method. The complexes were purified by FPLC in two steps: anion exchange (ResQ) followed by gel filtration (Sephadex S200). The yields of refolding and purification were around 2% for H-2K^k–SV40(560–568) complex and 4% for H-2K^k–HA(259–266) complex. The proteins eluted in 10 m*M* Tris, 50 m*M* NaCl pH 8 and were concentrated to 3 mg ml^{-1}, giving maximum overall yields of 1.5% for the nonapeptide complex and 2.8% for the octapeptide complex. The authenticity and purity of the complexes were checked by reducing SDS–PAGE gel and by mass spectrometry.

2.2. Crystallization

For each crystallization trial, batches of freshly purified protein were used. For the H-2K^k–HA(259–266) complex, an extensive screen (3 × 96 conditions) was conducted employing the sitting-drop vapour-diffusion method using a Cartesian nanodrop-dispensing robot. Crystals were obtained from the MDL II screen (Molecular Dimensions Ltd, condition 18) in 0.3 µl drops (0.2 µl 3 mg ml^{-1} protein solution plus 0.1 µl well solution). A scale-up using classical Linbro plates (2 µl 3 mg ml^{-1} protein solution plus 1 µl crystallization buffer) resulted in crystals suitable for data collection (100 × 100 × 400 µm in size; Fig. 1*a*). The final crystallization conditions were 4.3 *M* NaCl, 0.1 *M* HEPES pH 7.5. Crystals grew in few days using protein solution less than one week old.

For H-2K^k–SV40(560–568), an initial screen was performed based on MHC crystallization conditions from the literature. Crystals were obtained at 293 K by the hanging-drop vapour-diffusion method. The best crystals (drops consisting of 1–1.5 µl 6–3.7 mg ml^{-1} protein solution plus 1 µl well buffer) grew very slowly (weeks to months) in 1.6 *M* ammonium sulfate, 0.1 *M* MES pH 6.5 and additives (either 2–5% glycerol or 3% methyl pentanediol). They were microscopic needles resulting from hypernucleation. Two techniques were used to improve the quality of these crystals: homogeneous macroseeding followed by a stepwise increase (0.05 *M* intervals) in the ammonium sulfate concentration by transferring the cover slips holding the hanging drops. The dimensions of the final crystals were about 10 × 10 × 300 µm (Fig. 1*b*). The reproducibility of the crystallization appeared to be highly dependent on the age of the protein, as the use of solutions older than 3–4 d hampered crystal growth.

2.3. X-ray diffraction experiments

The H-2K^k–HA(259–266) crystals were soaked for several minutes in reservoir solution supplemented with 30% PEG 400 prior to flash-cooling in a nitrogen stream at 100 K (Oxford Cryosystems). It was observed that increasing the PEG concentration yielded more compact crystals (as evaluated from the determination of the unit-cell parameters) with better diffraction quality. X-ray diffraction data with a resolution of 2.5 Å were recorded from a single crystal at beamline ID14 EH-2 at the ESRF, Grenoble, France (λ = 0.9297 Å). The crystal belongs to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 111.76$, $c = 109.42$ Å. The volume of the unit cell is compatible with one molecule in the asymmetric unit. All data processing was performed using the *DENZO*/*SCALEPACK* programs (Otwinowski & Minor, 1997). The statistics of the data collection are given in Table 1.

The H-2K^k–SV40(560–568) crystals were soaked in a solution containing 30% glycerol, 1.6 *M* ammonium sulfate, 0.1 *M* MES pH 6.5 and then flash-cooled in a nitrogen stream at 100 K. Because of their size (about 10 µm in width), they had to be handled very carefully during the cryoprotection step. All data were collected using synchrotron radiation (ESRF, Grenoble, France, beamlines ID14 EH2 and EH4). As diffraction decay was observed arising from radiation damage, complete data sets were obtained by translating the needle-shaped crystal to target three different regions.

Different lattices (orthorhombic and monoclinic) were found for crystals of similar morphology that had been obtained with either 5% glycerol or 3% MPD as additives. The first complete data set was obtained from a crystal with a primitive orthorhombic lattice (a = 93.1, b = 102.4, c = 203.9 Å). The quality of the diffraction was fairly poor, with 77% of the reflections collected between 20 and 3.3 Å resolution. Systematic absences occurred on the b axis ($0k0$ with $k = 2n$) and c axis ($00l$ with $l = 2n$). Another data set was collected from a

Table 1
Data collection.

Values in parentheses are for the highest resolution shell.

	H-2K^k–HA(259–266)	H-2K^k–SV40(560–568)
Space group	$P3_221$	$P2$
Unit-cell parameters	$a = b = 111.76$, $c = 109.42$	$a = 85.36$, $b = 72.46$, $c = 88.665$, $\beta = 111.16$
Resolution limits (Å)	15.0–2.5 (2.64–2.50)	20.0–3.0 (3.05–3.00)
Unique reflections	27403	18302
Completeness (%)	99.3 (99.2)	90.2 (90.0)
Data with $I > 3\sigma(I)$ (%)	72.1 (37.3)	47.5 (14.6)
Redundancy	2.7 (2.7)	2.5 (2.5)
Average $I/\sigma(I)$	8.9 (2.1)	4.2 (1.7)
R_{sym}† (%)	6.9 (33.8)	15.8 (39.7)

† $R_{sym} = 100(\sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi})$.

crystal of similar morphology (also grown in 5% glycerol) that also had a primitive orthorhombic lattice, but with different unit-cell parameters (a = 73.1, b = 88.9, c = 159.6 Å). The quality of diffraction was improved, with a resolution of 2.9 Å (91.5% completeness). A third needle-shaped crystal grown in 3% MPD was found to belong to a monoclinic space group. An almost complete data set (89% completeness) was collected on ID14 EH2 beamline (ESRF, Grenoble, France) and have unit-cell parameters a = 85.13, b = 72.63, c = 88.79 Å, β = 111.24°. The statistics of the data collection are given in Table 1. No reflections were measured along the b axis, preventing space-group determination. The calculated Matthews coefficient suggested the presence of two molecules in the asymmetric unit.

3. Results and discussion

The mouse MHC molecule H-2K^k and β-2 microglobulin were expressed as inclusion bodies and refolded in the presence of octapeptides and nonapeptides. Five complexes were obtained, with overall yields of production in the range 1.5–3% dependent on the peptide length, and were crystallized. Two of these complexes, those with HA(259–266) and SV40(560–568) peptides, gave crystals of satisfactory quality, with crystals of H-2K^k–HA(259–266) generating higher quality data than those of H-2K^k–SV40(560–568).

The molecular-replacement method (using *AMoRe*; Navaza, 1994) was used to solve the structure of H-2K^k–HA(259–266), using the H-2K^b structure (PDB code 1leg) as the search model. The complex was separated into two independent sets of domains: $\alpha1/\alpha2$ and $\alpha3/\beta2$m. Solutions for both the rotation and the translation functions for the two sets of domains were obtained in space group $P3_221$ using data between 8.0 and 4.0 Å. Before fitting, the correlation coefficient and R factor were 23.1 and 48.8%, respectively, which refined to 38.4 and 44.5%, respectively. For H-2K^k–SV40(560–568), molecular replacement was tried with each of the three data sets and the structure of the octapeptide complex was used as search model. The complex was separated into the same independent sets of domains. Despite numerous trials, it was not possible to solve the structure using the data from the orthorhombic space groups. The structure was ultimately solved in the monoclinic space group $P2$ using the third data set. Two molecules were found in the asymmetric unit. Before fitting, the correlation coefficient and R factor were 31.8 and 50.0%, respectively, which refined to 40.3 and 46.3%, respectively. Although the space group $P2$ is rarely seen for protein crystals, another MHC, H-2L^d (PDB code 1ldp; Speir *et al.*, 1998), in complex with a mixture of peptides, crystallized in this space group. The refinement of the two structures is in progress.

We express our gratitude to Dr B. Malissen for his constant support. We thank the members of the ESRF staff for their help in taking diffraction measurements on beamline ID14.

References

Achour, A., Michaelsson, J., Harris, R. A., Odeberg, J., Grufman, P., Sandberg, J. K., Levitsky, V., Karre, K., Sandalova, T. & Schneider, G. (2002). *Immunity*, **17**, 757–768.
Achour, A., Persson, K., Harris, R. A., Sundback, J., Sentman, C. L., Lindqvist, Y., Schneider, G. & Karre, K. (1998). *Immunity*, **9**, 199–208.
Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. & Wilson, I. A. (1992). *Science*, **257**, 919–927.
Gould, K. G., Scotney, H. & Brownlee, G. G. (1991). *J. Virol.* **65**, 5401–5409.
Madden, D. R., Garboczi, D. N. & Wiley, D. C. (1993). *Cell*, **75**, 693–708.
Matsumura, M., Fremont, D. H., Peterson, P. A. & Wilson, I. A. (1992). *Science*, **257**, 927–934.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pullen, J. K., Horton, R. M., Cai, Z. L. & Pease, L. R. (1992). *J. Immunol.* **148**, 953–967.
Rawle, F. C., O'Connell, K. A., Geib, R. W., Roberts, B. & Gooding, L. R. (1988). *J. Immunol.* **141**, 2734–2739.
Smith, K. J., Reid, S. W., Harlos, K., McMichael, A. J., Stuart, D. I., Bell, J. I. & Jones, E. Y. (1996). *Immunity*, **4**, 215–228.
Speir, J. A., Garcia, K. C., Brunmark, A., Degano, M., Peterson, P. A., Teyton, L. & Wilson, I. A. (1998). *Immunity*, **8**, 553–562.
Townsend, A. & Bodmer, H. (1989). *Annu. Rev. Immunol.* **7**, 601–624.