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Folding, Heterodimeric Association and Specific Peptide Recognition of a Murine αβ T-cell Receptor Expressed in *Escherichia coli*

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In a systematic study of the murine T-cell receptor UZ3-4, expressed and refolded from inclusion bodies in *Escherichia coli*, it was found that functional molecules can be obtained only under a very narrow set of conditions. The refolded T-cell receptor UZ3-4 specifically recognizes its cognate peptide (from mycobacterial Hsp60) in the context of H-2D^b, but not another peptide bound to H-2D^b, and the dissociation constant was determined by BIAcore as 10^{-4} M. Using T-cell receptor constructs comprising all extracellular domains (VαCα and VβCβ), found to be necessary for stability of the final product, significant amounts of native molecules were obtained only if the intermolecular Cα-Cβ disulfide bridge bond was deleted, even though the interaction between the complete α and β-chain was determined to be very weak and fully reversible ($K_D \approx 10^{-7}$ to 10^{-6} M). Fusion of Jun and Fos to the constant domains also decreased the folding yield, because of premature association of intermediates leading to aggregation. Furthermore, only in a very narrow set of concentrations of oxidized and reduced glutathione, native disulfide bonds dominated. This shows that T-cell receptor domains are very prone to aggregation and misassociation during folding, compounded by incorrect disulfide bond formation. Once folded, however, the heterodimeric molecule is very stable and could be concentrated to millimolar concentration.

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Introduction

The T-cell receptor (TCR) plays the central role in cellular immunity, ultimately distinguishing self from non-self. The ability of T-lymphocytes to specifically recognize antigenic peptides in the context of the major histocompatibility complex (MHC) is due to the TCR, a heterodimer composed of an α-chain and a β-chain linked by a disulfide bridge and exposed at the surface of T-cells *via* membrane anchors. Their three-dimensional (3D) structure was predicted to have homology with antibody Fab fragments (Chothia *et al.*, 1988). This was confirmed by the structure determination of various extracellular portions of the TCR (Bentley *et al.*, 1995; Fields *et al.*, 1995; Garboczi *et al.*, 1996a; Garcia *et al.*, 1996a, 1998; Housset *et al.*, 1997; Ding *et al.*, 1998; Wang *et al.*, 1998). Although structures have been reported for TCR-peptide-MHC complexes (TCR-pMHC; Garboczi *et al.*, 1996a; Garcia *et al.*, 1996a, 1998; Ding *et al.*, 1998), the understanding of the structural basis of the recognition of the pMHC by the TCR is still very limited. Particularly, the narrow window of permissible affinities which results in the triggering of T-cells and the distinction between agonist and antagonist peptides, delivering either a stimulatory or an inhibitory signal to the T-cells, needs more detailed structural correlates. The degeneracy of the TCR specificity and its implication in autoimmune disorders is currently a matter of great interest and directly related to this energetic window. Therefore, we obviously need more structural information about TCR-pMHC complexes.

Abbreviations used: β_2m, β_2-microglobulin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidinium hydrochloride; GSH, L-glutathione, reduced form; GSSG, L-glutathione, oxidized form; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; pMHC, peptide-MHC complex; RT, room temperature; TBS, Tris-buffered saline; TCA, trichloroacetic acid; TCR, T-cell receptor; 3D, three-dimensional; scTCR, single-chain TCR.

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In spite of recent successes, the TCR has been very difficult to isolate and to purify in the quantities that are necessary for structural and functional characterization. Several approaches have been followed to produce soluble forms of the TCR (reviewed by Wülfing & Plückthun, 1995; Wilson & Garcia, 1997). In eukaryotic cells, these strategies consisted either of secretion of the receptor devoid of its transmembrane domains or its cleavage from an engineered linker (Gascoigne *et al*., 1987; Mariuzza & Winter, 1989; Lin *et al*., 1990; Gregoire *et al*., 1991; Engel *et al*., 1992; Weber *et al*., 1992; Chang *et al*., 1994; Chung *et al*., 1994; Corr *et al*., 1994; Kappler *et al*., 1994; Seth *et al*., 1994; Alam *et al*., 1996; Khandekar *et al*., 1997b). The TCRs produced in this way allowed functional characterization and placed the affinities of TCRs for pMHC (K_D ranging from 10^{-4} M to 10^{-6} M) far below the range of typical antibody-antigen interactions. However, the low-expression yields and the glycosylation when expressed in eukaryotic cells often prevented the growth of good quality crystals. This latter point led several groups to eliminate glycosylation sites (Bentley *et al*., 1995; Wang *et al*., 1998) or to desialylate the TCR produced by treating it with neuraminidase (Housset *et al*., 1997) to allow structural determination.

Escherichia coli is, in principle, an attractive alternative host to eukaryotic systems. Indeed, high-level production has been achieved for a great variety of proteins, and many unglycosylated proteins have been generated suitable for characterization and structural studies. Since TCRs share a structural homology with Fab fragments of antibodies, it was attractive to follow antibody expression strategies (Plückthun, 1992; Plückthun *et al*., 1996). Most attempts to produce TCRs in *E. coli* had consisted of the production of scTCRs, either functionally expressed or refolded *in vitro* (Novotny *et al*., 1991; Hoo *et al*., 1992; Ward, 1992; Kurucz *et al*., 1993; Hilyard *et al*., 1994; Wülfing & Plückthun, 1994; Reiter *et al*., 1995; Schlueter *et al*., 1996; Schodin *et al*., 1996; Khandekar *et al*., 1997a; Plaksin *et al*., 1997). Recently, the use of *E. coli* as an expression host for the production of the whole extracellular portion of the TCR has been reported for the human TCRs A6 and B7 (Garboczi *et al*., 1996a; Ding *et al*., 1998). These TCRs were obtained after *in vitro* refolding from inclusion bodies (Garboczi *et al*., 1996b) and were soluble enough to allow the structural determination of these TCRs in complex with pMHC.

Many strategies have been proposed to engineer soluble TCR fragments, such as single-chain TCR (scTCR), disulfide-linked Vα-Vβ (dsTCR; Reiter *et al*., 1995), three-domain scTCR Vα-linker-Vβ-Cβ (Chung *et al*., 1994), four-domain constructs (analogous to antibody Fab fragments) with and without interchain disulfide bond, or fusion of TCR chains to leucine zippers (Chang *et al*., 1994). However, attempts to refold TCRs *in vitro* or to express them functionally, whatever their sequences or formats, encountered great difficulties in many laboratories. For example, the solubility and stability of the scTCRs appeared considerably lower than scFv antibodies, explaining why few reports had presented any evidence of pMHC binding (Schlueter *et al*., 1996; Schodin *et al*., 1996; Khandekar *et al*., 1997a; Plaksin *et al*., 1997).

With the aim to develop a reliable system suitable for functional and structural studies of TCRs, we report here a systematic investigation of a high-level production of the αβ-heterodimer form of the murine TCR UZ3-4 (Schoel *et al*., 1994), corresponding to an antibody Fab fragment. It was expressed in *E. coli* and refolded *in vitro* from inclusion bodies, and we report its characterization and its affinity for the antigenic peptide determined by surface plasmon resonance. The highest yield of refolding was obtained with the Fab-like construct lacking the interchain disulfide bridge. The addition of Jun/Fos heterodimerization domains to C-terminal ends of TCR chains decreased the yield of refolding. We show also that the optimal conditions of refolding are limited to a rather narrow minimum in parameter space, and that α-chains and β-chains separately refolded are native by themselves and can simply be mixed to reconstitute the TCR. The refolded recombinant TCR lacking the interchain disulfide bridge is biologically active and specific, as it recognizes the agonist peptide with the sequence SALQNAASIA from the *Mycobacterium* Hsp60 (Schoel *et al*., 1994) presented in the context of MHC H-2D^b, but fails to recognize an irrelevant peptide (gp33) from LCMV which also binds MHC H-2D^b (Aichele *et al*., 1994). The dissociation constant for the TCR-pMHC complex was determined to be about 100 μM, which places the TCR UZ3-4 in the low range of affinities observed for TCRs. Once refolded, the native TCR UZ3-4 is very soluble (>1 mM) and its high yield of production makes it suitable for further structural studies.

Results and Discussion

General considerations

Preliminary experiments with the refolding of a series of TCR constructs of different T-cell clones (A.C.T., F.P. & A.P., unpublished results) have led us to the conclusion that no published method or format of the recombinant TCR (see above) appeared to be of general utility. Great differences between the aggregation tendencies of different TCRs also became apparent. We therefore decided to investigate one of the more promising candidates, the murine TCR specific for a mycobacterial Hsp60 peptide in the context of H-2D^b (Schoel *et al*., 1994) in a systematic fashion to elucidate the properties of these molecules. Because of hints of a gradual aggregation of scTCRs upon storage, at least in the ones we had investigated, the present study was carried out with heterodimers comprising all four extracellular domains (VαCα and VβCβ). As prior work on other TCRs has indicated that weak

chain association, incorrect disulfide formation and premature aggregation may all be problems, we systematically investigated the influence of redox potential, presence and absence of the interdomain Cα-Cβ disulfide bond or the presence and absence of the Jun and Fos leucine zippers to the C termini of the constant domains.

Expression and purification of TCR UZ3-4 subunits

The subunits (Figure 1) were produced separately in *E. coli* as inclusion bodies. The inclusion bodies were washed and then solubilized in Gdn-HCl (guanidinium hydrochloride), and any disulfide bridges were reduced by DTT. After desalting to remove DTT, the two subunits were purified to homogeneity (Figure 2, lane 4) under denaturing conditions by Ni-agarose chromatography using the hexahistidine tags on both chains, and their cysteine residues were kept in a reduced state for storage (1 mM DTT). The yield of purified protein was around 70 mg/l cell culture for each subunit. These high yields were only reached by co-transforming a plasmid overexpressing a rare tRNA together with the T7 expression plasmids (Brinkmann *et al*., 1989), since the TCR β-subunit contains several rare arginine codons that are problematic for expression in *E. coli* (Kane, 1995).

Refolding of TCR UZ3-4

As reported for the human A6 TCR (Garboczi *et al*., 1996b), it was possible to refold the murine TCR UZ3-4 either by dialysis (data not shown) or by rapid dilution. However, rapid dilution methods, when combined with pulse renaturation (Rudolph & Lilie, 1996), allow the renaturation of larger amounts of protein in an equal volume of buffer than direct renaturation by dialysis. Furthermore, an individual optimization of the redox potential was required, or an incorrect disulfide bond was favored.

We started our investigation using similar conditions as described by Garboczi *et al*. (1996b). The α and β-subunits were mixed in equimolar amounts prior to renaturation. Starting with this totally reduced and denatured material, TCR UZ3-4 was allowed to reach its native conformation by diluting the protein mixture into buffer containing 1 M arginine as a co-solvent, under conditions that allowed reshuffling of disulfide bridges. From experiments with different TCR sequences, we observed a large visible aggregation occurring rapidly when refolding TCRs in 0.4 M arginine that could be fully prevented by using higher arginine concentrations, such as 0.8 to 1.0 M. Thus, we used 1.0 M arginine for all subsequent refolding experiments.

After *in vitro* refolding, the majority of the refolded protein migrated faster than the reduced subunits, showing that intramolecular disulfide bonds had formed (Figure 2, lanes 1 and 4). However, a third species was observed on non-reducing SDS-PAGE (Figure 2, lane 1) after purification of refolded TCR UZ3-4 by gel filtration. This species corresponded to the α-subunit with an incorrect disulfide bridge (as determined by refolding isolated subunits; data not shown).

α-subunit

```
  1         10          20         30          40
  ¦         ¦           ¦         ¦ab         ¦
MDGDSVTQKEGLVTLTEGLPVMLNCTYQTIYSNAFLFWYVHYLNE
                                CDR1

          50         60         70         80
          ¦          ¦          ¦          ¦
SPRLLLKSSTDNKRTEHQGFHATLHKSSSSFHLQKSSAQLSDSAL
            CDR2

  90         100         110     C-domain
  ¦          ¦           ¦       ¦
YYCALSDRGRASSSFSKLVFGQGTSLSVVPNIQNPEPAVYQLKDP
           CDR3

RSQDSTLCLFTDFDSQINVPKTMESGTFITDKTVLDMKAMDSKSN

GAIAWSNQTSFTCQDIFKETNATYPSSDVPSGGEFHHHHHH
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β-subunit

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 1         10          20         30          40
 ¦         ¦           ¦          ¦          ¦
MEAAVTQSPRSKVAVTGGKVTLSCHQTNNHDYMYWYRQDTGHGLR
                                CDR1

      50         60         70         80          90
      ¦          ¦          ¦          ¦           ¦
LIHYSYVADSTEKGDIPDGYKASRPSQENFSLILELASLSQTAVY
           CDR2

           100  110       C-domain
           ¦    ¦         ¦
FCASSVDRTEVFFGKGTRLTVVEDLRNVTPPKVSLFEPSKAEIAN
       CDR3

KQKATLVCLARGFFPDHVELSWWVNGKEVHSGVSTDPQAYKESNY

SYSLSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDKWPEGSPKPV

TQNISAEAWGRADSGGEFHHHHHH
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Figure 1. Amino acid sequences of the expressed α and β-subunits of the TCR UZ3-4. The free cysteine at position 183 in Cβ was mutated to serine (represented in boldface within the sequence SY **S** LS), as were the respective cysteine residues involved in the interchain disulfide bridge (within the sequences VP **S** GG and AD **S** GG for the α and β-subunits, respectively). The N-terminal methionine residues were added for bacterial expression. The numbering of residues and the assignment of complementarity determining regions (represented as underlined boldface characters) are as described by Kabat *et al*. (1991).

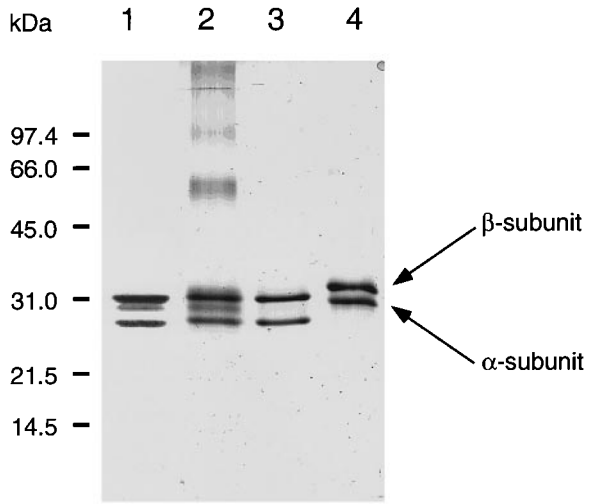

Figure 2. Non-reducing SDS-PAGE analysis of the TCR at different stages of production. Lane 1, TCR UZ3-4 refolded in 1 M arginine buffer (GSH = 6.3 mM, GSSG = 3.7 mM), purified by gel filtration chromatography; lane 2, TCR UZ3-4 in 1 M arginine buffer after refolding (GSH = 0.2 mM, GSSG = 0.2 mM); lane 3, TCR UZ3-4 refolded (GSH = 0.2 mM, GSSG = 0.2 mM), purified by gel filtration chromatography and concentrated in TBS; lane 4, for comparison, denatured and reduced subunits purified by Ni-agarose chromatography.

In order to improve refolding conditions by rapid dilution and to minimize this contaminant, a systematic study of the effect of the redox potential on the distribution of disulfide-bridged species was carried out. For this purpose, small-scale refolding experiments were performed as described in Materials and Methods using 36 different redox potential conditions. Refolded protein was directly analyzed on non-reducing SDS-PAGE. As shown in Figure 3, the two major bands corresponding to α and β-subunits with the native disulfide bridges were favored only at concentrations of the reduced form of L-glutathione (GSH) $\leqslant$1 mM and the oxidized form of L-glutatione $\leqslant$1 mM. Scanning of gels and integration of the bands showed that the native bonds are maximized at the expense of incorrectly folded subunits when GSH and GSSG were both at 0.2 mM. Therefore, these redox conditions were chosen to routinely produce the TCR UZ3-4 on a large scale. Interestingly, similar equilibrium redox potentials, which are proportional to $\ln([GSH]^2/[GSSG])$, are obtained at several other combinations of GSH and GSSG, but the folding yield was lower than at 0.2 mM for each GSH and GSSG. This suggests that kinetic factors may also be of importance.

Purification of native TCR UZ3-4

After large-scale refolding in 1 M arginine, the state of the refolded protein was checked on a non-reducing gel (Figure 2, lane 2) showing a higher degree of homogeneity in disulfide-bridged species than before optimizing the redox potential. The arginine used as co-solvent to break up weak non-covalent interactions was exchanged by dialysis against Tris-buffered saline (TBS), and then the soluble material was concentrated and loaded on a preparative S200HR gel filtration column. The

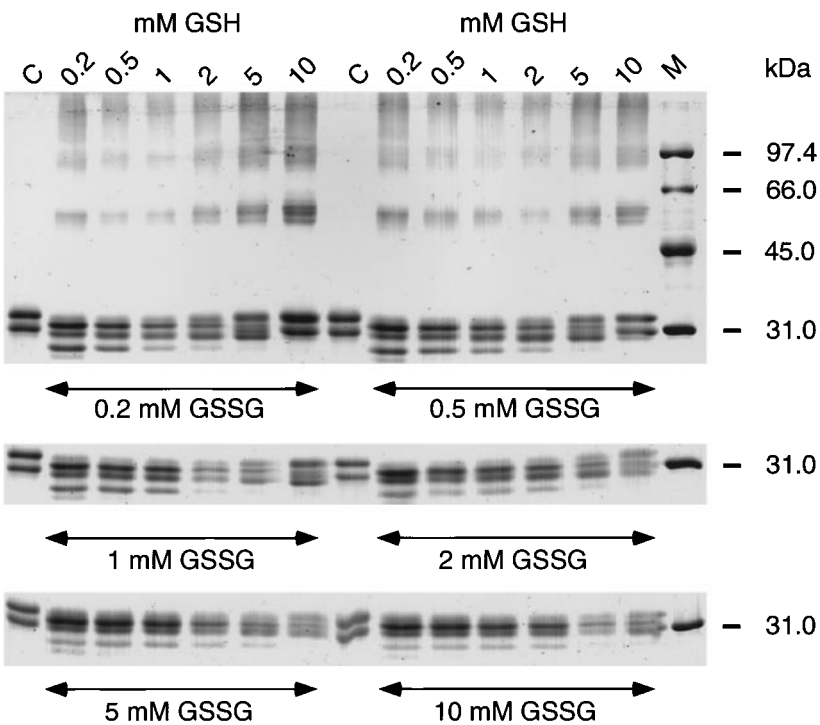

Figure 3. Optimization of redox conditions used to refold the TCR UZ3-4. Equimolar amounts of denatured and reduced subunits were refolded for 48 hours at 4 °C in 1 M arginine buffer (pH 8.0) containing the indicated concentrations of GSH and GSSG. After precipitation by TCA, the distribution of disulfide-bridged species was analyzed by non-reducing SDS-12 %PAGE. Lane C, for comparison, reduced α and β-subunits; M, molecular mass markers.

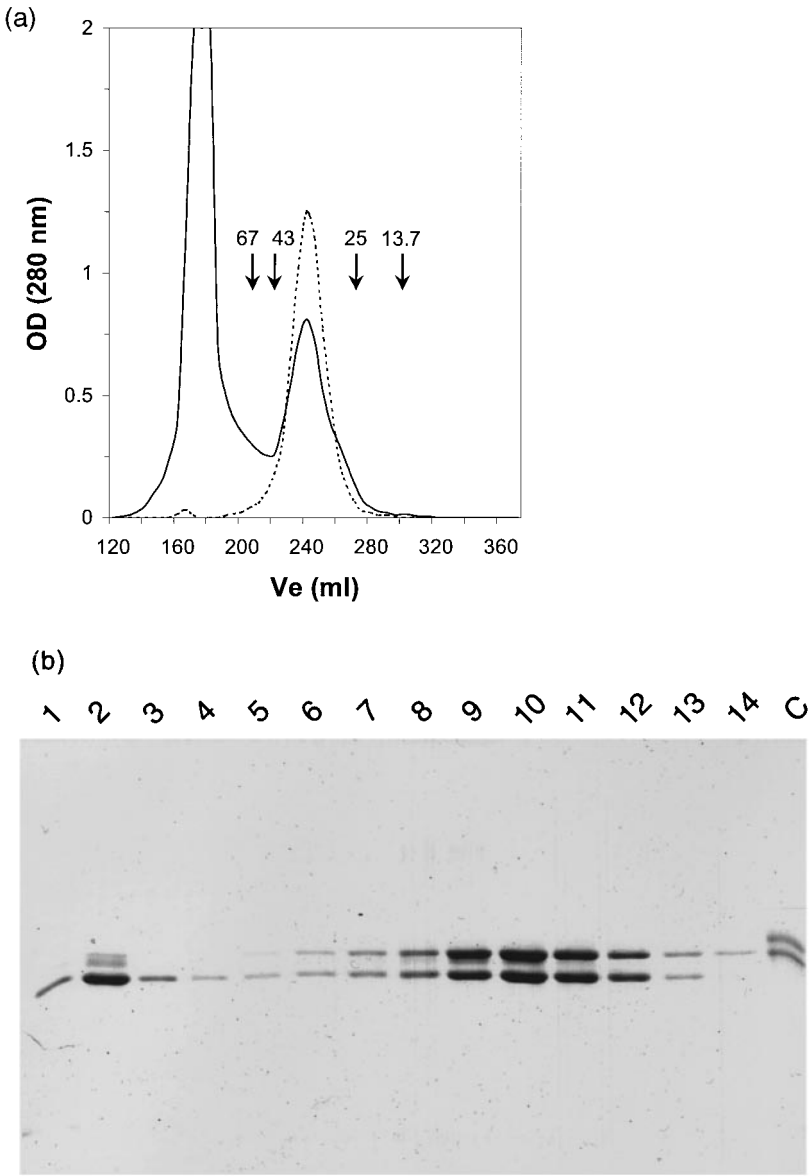

Figure 4. (a) Gel filtration chromatography profiles of refolded TCR UZ3-4 after the purification (continuous line) and reloading of the peak (220-270 ml) on an S200HR column (broken line). The column was calibrated with the indicated molecular mass markers (in kDa). (b) SDS-PAGE analysis of the broken-line peak. Each lane from 1 to 14 corresponds with a 10 ml fraction in the range 150 ml-280 ml from the elution profile. Lane C, control with α and β-subunits reduced.

elution profile of the refolded TCR UZ3-4 (Figure 4(a)) showed two peaks. The first peak eluted in the void volume, indicating aggregated protein. The second peak corresponded to a protein with an apparent molecular mass of ≈39 kDa. This is in the range expected for refolded TCR UZ3-4, but less than the theoretical molecular mass of the αβ-heterodimer (≈52.7 kDa). As discussed for the gel filtration experiments with the β-subunit alone (see below), this behavior seems to be due to interactions between this subunit and the matrix of the gel (Figure 5). In addition, a shoulder observed at 265 ml corresponded to the α-subunit.

Fractions from the peak at about 39 kDa were pooled, concentrated and further purified by preparative gel filtration (Figure 4(a)) and analyzed by SDS-PAGE (Figure 4(b)). The major peak eluted from this second run of purification was symmetric and contained pure αβ-heterodimers formed by homogeneously folded subunits, as assessed by non-reducing gels (Figure 4(b), lanes 9-11; Figure 2, lane 3). Indeed, the shoulder observed previously was no longer visible, and the α-subunit contamination appeared as a peak eluting in the void volume (Figure 4(b), lanes 1-3), showing that the α-subunit is less stable than the αβ-heterodimer upon concentration and tends to aggregate. The αβ-heterodimers are not covalently linked in this TCR UZ3-4 construct, which does not have the terminal interchain disulfide bridge. The separation of both subunits on non-denaturing PAGE also demonstrates that there is no incorrect interdomain disulfide bond formed.

Scanning of the SDS/polyacrylamide gel (Figure 2, lane 3) showed that α and β-subunits were present in equimolar amounts, indicating the correct association of the two chains. It was possible to concentrate this purified TCR UZ3-4 at least up to 1 mM without aggregation according to gel filtration experiments carried out even after one

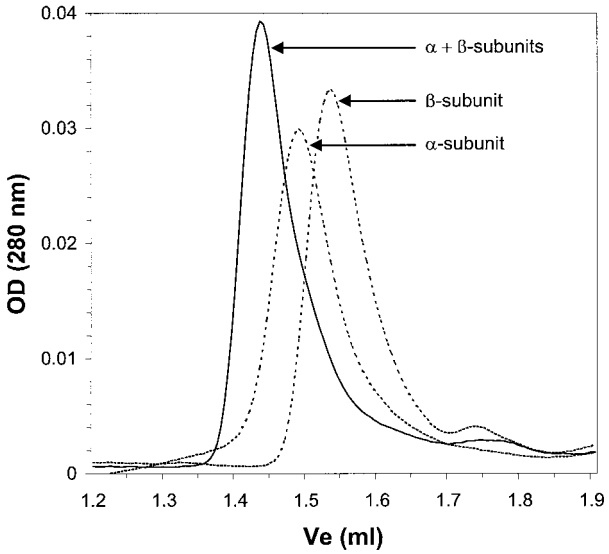

Figure 5. Elution profiles for α-subunit and β-subunit refolded separately (injected at 20 μM, broken lines). Equal amounts of α-subunit and of β-subunit (50 μl) were mixed and incubated for one hour at 4 °C prior to injection (continuous line).

month storage (data not shown). The overall yield of purified TCR UZ3-4 was about 8% of the protein material used for refolding, which corresponds to about 3 to 4 mg per liter of bacterial culture. Such yields, in conjunction with the stability of the TCR UZ3-4, opened the road to biophysical and structural studies.

Characterization of the TCR UZ3-4

The purified and refolded TCR UZ3-4 was analyzed by mass spectrometry (results not shown), and the molecular masses ($\alpha = 24{,}614.3$ Da, $\beta = 28{,}078.6$ Da) were in good agreement with the theoretical values ($\alpha = 24{,}613.3$ Da, $\beta = 28{,}078.1$ Da).

To rule out that the peak observed on size exclusion chromatography could correspond to a mixture of homodimers (i.e. $\alpha\alpha + \beta\beta$) and not to the desired heterodimers (this cannot be resolved by SDS-PAGE analysis) we refolded and purified both subunits separately. We then analyzed the migration behavior of isolated α and β-subunits and also of α-subunit incubated with β-subunit for one hour at 4 °C prior to injection on a Superose-12 gel filtration column. As shown in Figure 5, each isolated subunit eluted as a single peak. When equimolar amounts of the two subunits were mixed and incubated for one hour at 4 °C prior to injection, a third species eluted from the column with an apparent molecular mass of about 45 kDa. This experiment clearly showed that neither subunit forms homodimers under these conditions, and even when diluted by a factor of 2 upon mixing, the refolded subunits were able to associate rapidly to form heterodimers. This excluded the possibility of a mixture of homodimers and implies that the interface between separated α and β-subunits is native to allow their spontaneous association into heterodimers. This result also implies that there is no conformational barrier in the isolated subunits that could prevent their association.

According to the respective theoretical molecular masses ($\alpha = 24.6$ kDa, $\beta = 28.1$ kDa), the β-subunit should elute before the α-subunit. However, this was not the case on a Superose-12 column (Figure 5) or on a S200HR column during the purification of isolated subunits. This could explain why the apparent molecular mass of the refolded TCR UZ3-4 is lower than expected. Indeed, it is likely that the interactions of the β-subunit with the column matrix, resulting in its retention, may also occur in the heterodimer.

At micromolar concentrations, αβ-heterodimers are formed quantitatively (Figure 5) in the absence of the interchain disulfide bond. Since refolding was much more efficient in the absence of this disulfide bond, we wanted to investigate at which concentration the chains would start to dissociate. The effect of the dilution on the stability of αβ-heterodimers was analyzed by size-exclusion chromatography after overnight incubation of αβ-heterodimers at different concentrations as described in Materials and Methods. As shown in Figure 6(a), retention times increased, and the height of the peaks decreased and became asymmetric with an extended trailing edge as the concentration of injected TCR UZ3-4 decreased. These observations are characteristic of a fast equilibrium (Stevens, 1989; Stevens *et al.*, 1995) between αβ-heterodimers and dissociated subunits. A plot of partition coefficients (K_{AV}) *versus* concentration of TCR UZ3-4 injected showed that at concentrations under 10 μM retention times increased significantly (Figure 6(b)). From these values an estimated dissociation constant of around 1 μM was obtained. Taking into account the dilution of proteins in the column (as the equilibrium is fast), the real K_D is likely to be between 1 and 0.1μM. Considering the large interaction surface between Vα and Vβ and Cα and Cβ, this dissociation constant is quite high, especially when compared with the corresponding K_D observed for heavy and light chains in Fab fragments (10^{-10} to 10^{-9} M; Bigelow *et al.*, 1974; Horne *et al.*, 1982) which are structurally related to the TCR. The K_D observed here is even at the low end of the K_D values usually observed for Fv fragments which dissociate into V_H and V_L (from 10^{-9} to 10^{-6} M; Hochman *et al.*, 1976; Klein *et al.*, 1979; Horne *et al.*, 1982; Glockshuber *et al.*, 1990; Anthony *et al.*, 1992; Polymenis & Stollar, 1995; Mallender *et al.*, 1996). This weak affinity between the α and β-chains may be related to the more polar constant domain interface in the TCRs compared with the more hydrophobic one in antibodies (Chothia *et al.*, 1988; Bentley *et al.*, 1995; Garboczi *et al.*, 1996a; Garcia *et al.*, 1996a). Based on the homogeneity observed for the refolded subunits in the αβ-heterodimers (Figure 2, lane 3), the

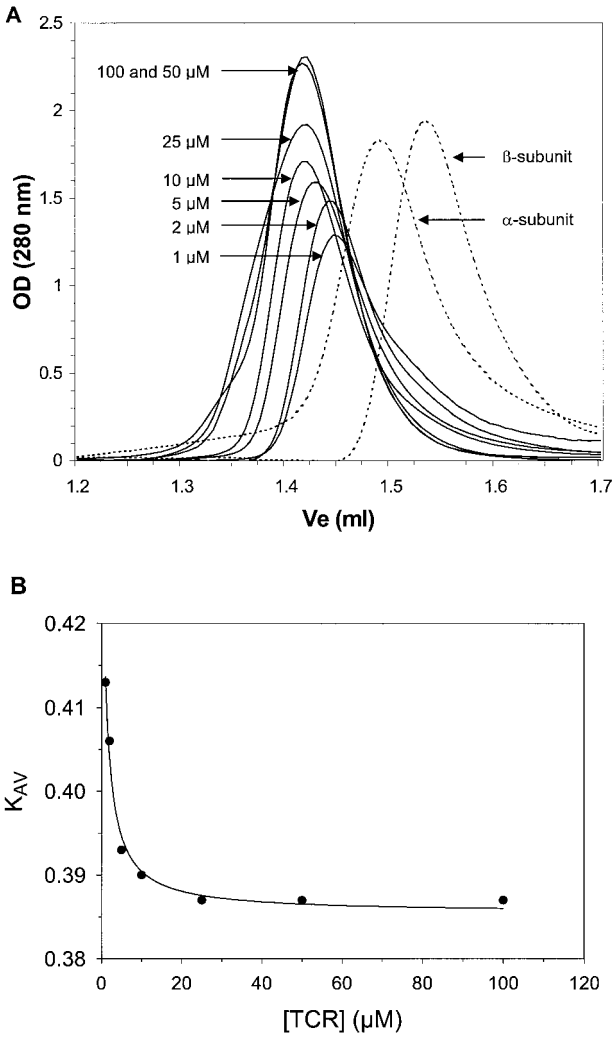

Figure 6. (a) Elution profiles for TCR UZ3-4 injected on Superose-12 column at 100, 50, 25, 10, 5, 2 and 1 µM (plotted at 1X, 2X, 4X, 10X, 20X, 50X, 100X, respectively). The α-subunit and β-subunit refolded separately were also injected (broken line). (b) plot of K_{AV} *versus* TCR concentration.

symmetric peaks of the heterodimers and both individual subunits (Figure 4 and 6) and the stability of the TCR UZ3-4 at high concentration (>1 mM) against aggregation, it is very likely that reversible native interactions occur between Vα and Vβ and the Cα and Cβ domains, even in the absence of the terminal disulfide bridge.

Since the interaction energy between the whole α-chain and the whole β-chain is already so weak, it will be even weaker between Vα and Vβ without the presence of constant domains, which is analogous to the difference between Fab and Fv constructs (Glockshuber *et al.*, 1990). It was briefly reported that co-expressed Vα and Vβ domains derived from the 1934.4 hybridoma could not associate (Ward, 1992). The weak association will also be found in scTCR fragments, and presumably the domains will dynamically shift between open and closed forms. Thus, the problem of very weak domain pairing could be one of the sources of trouble often reported about the stability of scTCR upon concentration (Novotny *et al.*, 1991; Hoo *et al.*, 1992; Kurucz *et al.*, 1993; Wülfing & Plückthun, 1994; Schlueter *et al.*, 1996; Schodin *et al.*, 1996). Further supporting this hypothesis, Reiter *et al.* (1995) showed that the stability of a scTCR fused to the toxin PE38 was significantly improved with the introduction of a disulfide bridge between the variable domains, suggesting that the linker may not be sufficient to maintain the domains associated. Additionally, exposure of the larger interaction surfaces between V domains and C domains observed in TCRs, compared with Fab fragments (Wang *et al.*, 1998; A. Honegger, personal communication), could also contribute to aggregation problems associated with scTCR molecules.

The weak interaction within the TCR UZ3-4 and the dissociation of α and β-subunits has been also observed for the A6 TCR and B7 TCR (Garboczi *et al.*, 1996b; Ding *et al.*, 1998). Obviously this dissociation is observable only because of the deletion of the terminal disulfide bridge in these three constructs, but even in its presence a dynamic opening and closing would be expected. The absence of the glycosylation in the Cα domain, which was shown to interact with the Cβ domain in the structure of a murine TCR (Garcia *et al.*, 1996a), may also cause the loss of some interaction energy, and this needs to be further explored. Attempts to refold the TCR UZ3-4 with the interchain disulfide bridge led to large amounts of covalent aggregates and to heterogeneity in the disulfide bridges (data not shown), consistent with the report for the TCR A6 (Garboczi *et al.*, 1996b). The addition of Jun and Fos heterodimerization domains to the C termini of TCR UZ3-4 subunits (with or without an interchain disulfide bridge) led to lower yields of refolding (data not shown). However, careful adjustment of the pH, away from the isoelectric points of either chain, could improve Jun/Fos folding (F.P., A.C.T. and A.P., unpublished results). Among the four Fab-like constructs of TCR UZ3-4 we investigated (with and without interchain disulfide bridge, with or without Jun/Fos), the one lacking the interchain disulfide bridge and lacking Jun/Fos refolded with the best yields (≈8 %). Furthermore, it was necessary to optimize the redox potential in order to obtain homogeneous TCR after refolding and purification. These results reflect the difficulties in obtaining soluble TCR fragments, and also the necessity to study carefully the process of refolding for each TCR.

The combination of the optimization of the expression of the construct of the refolding conditions and the high yields of bacterial expression allowed us to produce large amounts of soluble TCR fragment. With these large quantities of TCR, it is possible to overcome the problem of dissociation of subunits observed for the construct

presented here, by working at high concentrations of TCR, in order to characterize its function.

Binding of soluble TCR UZ3-4 to MHC H-2D^b complex

To analyze further the native state of refolded TCR UZ3-4, we tested it for specific binding to pMHC complexes. Since the TCR UZ3-4 dissociated into α and β-chains at low concentrations, the pMHC complexes were immobilized *via* the classic amine coupling strategy on a biosensor chip. The surfaces thus prepared were then exposed to TCR UZ3-4.

As shown in Figure 7, the TCR UZ3-4 bound to the specific pMHC (myhsp60 peptide) but failed to recognize a non-specific pMHC (gp33 peptide) which is bound by the same MHC H-2D^b. The signal obtained for a deactivated surface (not containing any protein) was the same as the signal observed for the surface prepared with non-specific MHC/peptide. Furthermore, injection of bovine serum albumin on specific and unspecific surfaces resulted in two superimposable sensorgrams (data not shown). Thus, the differentially higher signal only obtained for the specific pMHC surfaces, but not for any of the control injections, must be due to the recognition of the relevant pMHC by the refolded TCR UZ3-4.

As reported for other TCR/pMHC systems (Matsui *et al*., 1994; Plaksin *et al*., 1997) the on-rate and off-rate observed here are very fast, preventing us from analyzing the kinetic parameters. However, the availability of large amounts of correctly folded TCR and its stability at high concentrations enabled us to determine the dissociation constant at equilibrium. Increasing concentrations of soluble TCR UZ3-4 were injected (Figure 7) and the differences between specific and non-specific surfaces were plotted *versus* TCR UZ3-4 concentration (Figure 7(b)). These values gave a calculated dissociation constant of 109 (±16) μM. This value is the first K_D determination reported so far for a Fab-like TCR refolded *in vitro*.

The affinities of TCRs for MHC class I and class II molecules have been shown to range from 0.1 μM to 300 μM (Alam *et al*., 1996; Eisen *et al*., 1996; Garcia *et al*., 1997). Recently, a T-cell clone (UZ3-4) was established from mice immunized with mycobacterial Hsp 60 (Zügel *et al*., 1994). With a K_D value of about 100 μM, the TCR UZ3-4 is placed within the weak affinity range. Schoel *et al*. (1994) demonstrated in cellular assays that 100 pM of the peptide myhsp60 was sufficient to induce 50% lysis of target cells by CTL UZ3-4.

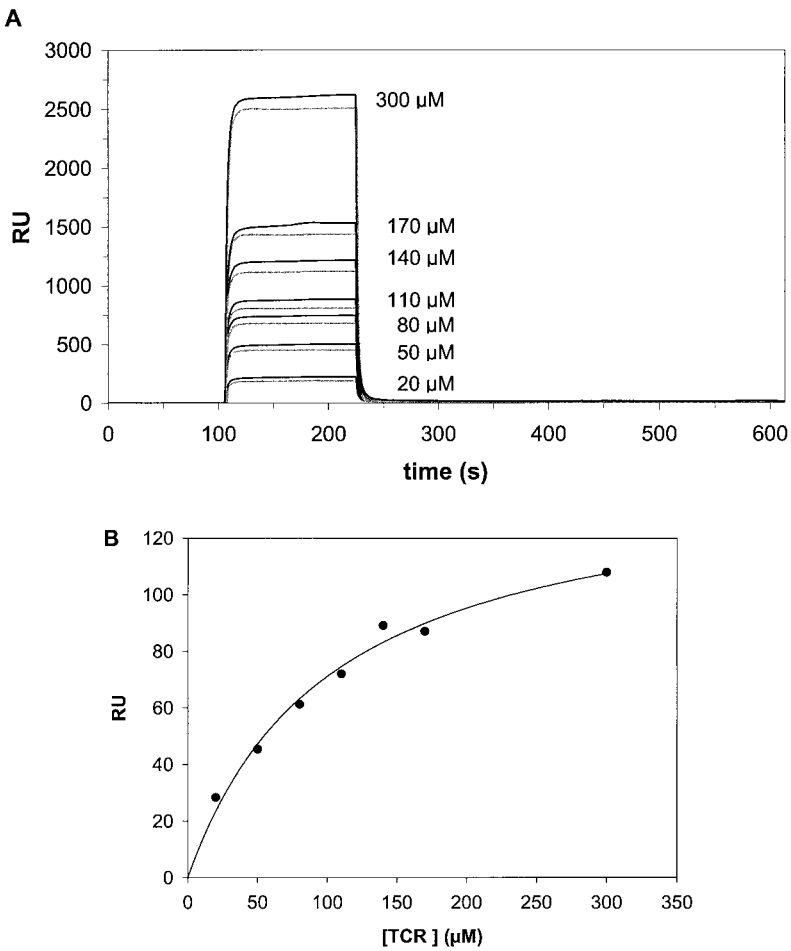

Figure 7. (a) BIAcore analysis of TCR UZ3-4 binding to immobilized pMHC complex. Two surfaces were prepared with ≈2500 RU gp33 pMHC (grey lines) and myhsp60 pMHC (black lines) immobilized to the sensor chip by amine coupling. The TCR UZ3-4 was injected at the indicated concentrations. (b) Differences between signals of specific and non-specific pMHC, obtained after two minutes injection for the two surfaces as a function of TCR concentration (measurements were averaged over at least two experiments). Other control surfaces (not-derivatized surface, bovine serum albumin injections other all surfaces) were indistinguishable from gp33 pMHC (data not shown).

Thus, our results further support that even a weak affinity between TCR and pMHC can be associated with a strong functional response *in vivo* (Sykulev *et al*., 1994; al-Ramadi *et al*., 1995). The intrinsic affinity measured here is probably lower than in a cellular context, since it was reported that the engagement of the co-receptor CD8 increases the affinity of TCR-complex for pMHC (Garcia *et al*., 1996b). A role of CD8 was also suggested from a study that pointed out discrepancies between the apparent affinity for TCR/pMHC and biological function (al-Ramadi *et al*., 1995). In spite of the weak affinities observed for the TCR/pMHC system, TCRs seem to be able to exhibit a striking degree of specificity by distinguishing, for example, two pMHCs that differ by just a phenylalanine to tyrosine substitution in the presented peptide (Sykulev *et al*., 1994). On the other hand, it was shown for numerous TCRs that a given clone is able to recognize various peptides even in different MHC contexts.

How specific and stringent the TCR recognition is may now be addressed *in vitro* with the soluble TCR UZ3-4. Indeed, our results show that the refolded TCR UZ3-4 is highly soluble and can be produced in large amounts with the system described here. This system allowed us to report the first binding constant determined for a refolded TCR in a Fab-like form. Furthermore, we showed that the recognition of the relevant pMHC does not require either the presence of the interchain disulfide bridge or glycosylation of the TCR αβ-heterodimer. This latter observation is in agreement with a report showing that affinities and even kinetics of the 2C TCR to its pMHC ligands appeared to be unchanged after deglycosylation (Garcia *et al*., 1997). These properties make it particularly suitable for further structural characterization and 3D structure determination.

Conclusions

Once the TCR is folded and at sufficient concentration, it behaves like a soluble, stable heterodimer of the α and β-chains with complete antigen specificity. Surprisingly, the association of both complete chains is very weak. However, heterodimer formation appears to have no particular activation barrier, as both refolded chains can simply be mixed, and must then be in rapid equilibrium. This highlights important differences from the much more strongly associated homologous Fab fragments of antibodies. Unfortunately, the folding pathway has many branches leading to aggregates, made further irreversible by very facile incorrect disulfide bond formation. We believe that this is the reason why the addition of a C-terminal interchain disulfide or, to a lesser extent, Jun/Fos failed to improve the yield of heterodimers, as they favor the off-pathway aggregation even more. Nevertheless, the molecule once refolded from *E. coli* inclusion bodies is completely antigen-specific, even though the affinity is weak ($K_D = 10^{-4}$ M). The poor physicochemical properties of TCRs may reflect the low selection pressure on weakly expressed surface bound molecules. Furthermore, the weak intrinsic affinity of the two chains for each other may confer flexibility to antigen recognition and influence the kinetics and affinity for antigen. This might even be a means to avoid too high a baseline affinity to the MHC molecule, independent of peptide context.

Materials and Methods

Construction of expression plasmids

The V-regions for the TCR UZ3-4 were cloned from mRNA by 5′ RACE (F.P., U. Steinhoff, U. Klemm, S. H. E. Kaufmann & A.P., unpublished results), and the cDNA so obtained was sequenced and served as the template for PCR to construct the expression plasmids. Separate α and β expression constructs were prepared by PCR amplification of TCR UZ3-4 cDNA as follows. An initiating methionine was inserted just before the first residue found in the mature α8 chain (Gregoire *et al*., 1996) and the β8.1 chain. The TCR sequences were included up to the cysteine residues involved in the interchain disulfide bridge at the C terminus of the constant domains and were extended by a sequence constituting a linker (GGEF) encoding an *Eco*RI site. The intrinsic *Nco*I site in each constant domain and the *Hin*dIII site in the Cα domain were removed, and the unpaired cysteine residue in the Cβ domain was changed to serine (Figure 1). A *Nco*I site and an *Eco*RI site were introduced at the 5′ and 3′ ends of both chains, respectively. The engineered α and β coding regions of the TCR were then ligated, using the *Nco*I and *Eco*RI sites, into the plasmid pTFT74 (Freund *et al*., 1993) carrying the T7 polymerase promotor and containing a His-tag as an *Eco*RI-*Hin*dIII cassette. The *E. coli* BL21(DE3)plysS strain (Studier *et al*., 1990) was used as expression host. The plasmid pUBS520 (Brinkmann *et al*., 1989) encoding an arginine-specific tRNA was co-transformed with the TCR UZ3-4 plasmids to allow high-level production of TCR subunits, since their coding sequences contain several AGA and AGG arginine rare codons.

Inclusion body production and purification

A 50 ml overnight culture of LB inoculated with a single bacterial colony was diluted into one liter of SB medium containing 0.5% (w/v) glucose, 100 μg/ml ampicillin, 30 μg/ml chloramphenicol and 50 μg/ml kanamycin. Bacteria were grown to midlogarithmic phase ($A_{550} = 0.6$-0.8) at 37 °C, and TCR production was induced by addition of 1 mM isopropyl β-D-thiogalactoside. After induction for four hours, the cells were harvested by centrifugation. Pelleted cells were resuspended in TE buffer (50 mM Tris, 1 mM EDTA (pH 8.0)) and sonicated for five minutes on ice. After the viscosity decreased, cell lysis was completed with a French press. Insoluble material was pelleted at 39,000 *g*, washed with TE buffer, pelleted, suspended in TE buffer containing 0.5% Triton X-100 and centrifuged. The inclusion bodies were finally resuspended in and pelleted from TE buffer containing 0.5 M Gdn-HCl.

Inclusion bodies were then solubilized with stirring overnight at RT (room temperature) in TE buffer containing 6 M Gdn-HCl and 100 mM DTT. Protein solutions were filtered and then desalted on a G-25 column equilibrated with 50 mM Tris buffer (pH 8.0) at RT, containing 6 M Gdn-HCl and 10 mM imidazole. The histidine-tagged TCR subunits were further purified by Ni-agarose (Ni-NTA agarose; Qiagen) affinity chromatography at a flow rate of 1 ml per minute. The Ni-agarose column was washed with 50 mM Tris buffer (pH 8.0) at RT, containing 6 M Gdn-HCl and 10 mM imidazole, and the protein was then eluted with the same buffer containing 250 mM imidazole. Protein concentrations were determined by spectrometry using extinction coefficients at 280 nm of 21,620 M^{-1} cm^{-1} and 46,940 M^{-1} cm^{-1} for the α-subunit and β-subunit, respectively (calculated from their sequences according to Gill & von Hippel 1989). The yield of purified protein was about 70 mg/l of bacterial culture.

Small scale refolding and optimization of redox potential

Equimolar amounts of denatured and reduced subunits were mixed in 50 mM Tris buffer containing 6 M Gdn-HCl and 1 mM DTT. The refolding was initiated at 4 °C by rapid dilution of aliquots of this protein mixture (final TCR concentration ≈80 μg/ml) in 36 tubes containing 1 ml of refolding buffer composed of 1 M L-arginine, 100 mM Tris, 2 mM EDTA (pH 8.0) at 4 °C with a defined redox potential. The redox potential, which is proportional to $\ln([GSH]^2/[GSSG])$, was adjusted using combinations of the following final concentrations of GSH and GSSG: 0.2/0.5/1.0/2.0/5.0/10.0 mM (i.e. 36 possibilities). After incubation at 4 °C for 48 hours with agitation, samples were treated with iodoacetamide (50 mM final concentration) for 15 minutes. Proteins were precipitated with 5% (w/v) TCA (final concentration) and centrifuged for 15 minutes at 16,000 *g*. Pellets were suspended in SDS-PAGE loading buffer and boiled for one minute prior to loading on non-reducing SDS-12%PAGE gels. Gels were stained with Coomasie brillant blue R250 and scanned to analyze the distribution of disulfide-bridged species.

Refolding of the TCR and purification

The denatured and reduced α-subunit (≈108 mg) and β-subunit (≈123 mg) were mixed in 50 mM Tris buffer (pH 8.0), 6 M Gdn-HCl, 1 mM DTT at RT to a final volume of 50 ml. One-third of this TCR mixture was refolded by rapid dilution with stirring in one liter of 100 mM Tris buffer (pH 8.0) containing 1 M L-arginine, 2 mM EDTA, 0.2 mM GSH and 0.2 mM GSSG at 4 °C for 10 to 16 hours. Then, another third of the TCR mixture was added after 10 and 16 hours to refolding buffer, respectively. The refolding was finally completed with an additional incubation at 4 °C for 24 hours. The refolding mixture was then dialyzed (M_r cutoff = 6000-8000) twice against 10 l of 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl at 4 °C. The protein solution was concentrated with an Amicon 8400 concentrator. Insoluble material was eliminated by centrifugation (20 minutes at 39,000 *g*) and filtration (0.22 μm pore size). The TCR was loaded on a Sephacryl S200HR column (2.5 cm × 100 cm) equilibrated in 20 mM Tris buffer containing 150 mM NaCl at a flow rate of 2.5 ml per minute at 4 °C. The fractions containing the αβ-dimer were pooled and concentrated by centrifugation (Ultra-free 15, Millipore) and further purified with a second round of gel filtration under the same conditions as described above. Heterodimer-containing fractions were identified by non-reducing SDS-12%PAGE. The TCR UZ3-4 was then concentrated up to 50 mg/ml and kept for storage at 4 °C in the buffer used for gel filtration. The yield of purified protein varied from 3 to 4 mg/l of bacterial culture. TCR subunits were also refolded and purified separately using the same protocols as described above.

Analytical gel filtration experiments

Gel filtrations were carried out at 20 °C with a SMART-system (Pharmacia) and a Superose-12 PC 3.2/30 column equilibrated in 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl at a flow-rate of 60 μl per minute. Starting from a stock solution of refolded TCR UZ3-4 of 100 μM, the protein was diluted in TBS to 100, 50, 25, 10, 5, 2 and 1 μM and incubated overnight at 4 °C. Fifty microliters of each dilution was injected on the column. The α and β-subunits was injected separately at 20 μM. Elution times were determined using the SMART Manager 1.50 software.

Peptides

Peptides were synthesized by Chiron Technologies to 95% purity and checked for molecular mass by mass spectrometry analysis. The synthetic peptides had the following sequences: SALQNAASIA (*Mycobacterium* Hsp 60 (myhsp60) peptide; Schoel *et al*., 1994) and KAVYNFATM (LCMV (gp33) peptide; Pircher *et al*., 1987).

Preparation of H-2D^b complex

Plasmids encoding H-2D^b or β_2-microglobulin (β_2m; Young *et al*., 1994) were obtained from Dr J. C. Sacchettini (Department of Biochemistry, Albert Einstein College of Medicine, New York). The growth of *E. coli* BL21(DE3)plysS expressing H-2D^b or β_2m and inclusion body preparations were carried out as described for the production of TCR UZ3-4. The washed inclusion bodies were separately solubilized in TE buffer containing 6 M Gdn-HCl and 100 mM DTT with stirring overnight at RT. Protein solutions were filtered and desalted on a G-25 column equilibrated with 50 mM Tris buffer (pH 8.0) containing 6 M Gdn-HCl at RT. The denatured and reduced H-2D^b subunit (≈32 mg) and β_2m subunit (≈12 mg) were mixed in 50 mM Tris buffer (pH 8.0), 6 M Gdn-HCl, 1 mM DTT at RT. A liter of refolding buffer consisting of 100 mM Tris buffer, 1 M L-arginine, 2 mM EDTA, 0.2 mM GSH, 1 mM GSSG containing 10 mg of myhsp60 or gp33 peptide (dissolved in 6 M Gdn-HCl) was cooled to 4 °C and the pH adjusted to 8.0. The MHC and β_2m were refolded by rapid dilution in this buffer with stirring at 4 °C for 48 hours. The protein solution was concentrated to about 100 ml with an Amicon 8400 concentrator and then dialyzed twice against one liter of 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl at 4 °C. After elimination of insoluble material by centrifugation (20 minutes, 39,000 *g*) and filtration (0.22 μm pore size), the peptide-H-2D^b complex was purified at 4 °C by gel filtration under the same conditions as described for the TCR UZ3-4. The purified pMHC complex was concentrated up to 10 mg/ml and kept for storage at 4 °C in the buffer used for gel filtration.

Surface plasmon resonance (SPR)

All experiments were carried out on a BIAcore instrument. Soluble peptide-H-2D^b complexes were immobilized on a CM5 chip by standard amine coupling chemistry by first activating the carboxy-dextran layer with a mixture of 0.05 M *N*-hydroxysuccinimide and 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. MHC H-2D^b (100 μg/ml) in 10 mM Na-acetate (pH 4.5) was then coupled *via* its amino groups. Unreacted surfaces were then inactivated with 1 M ethanolamine (pH 8.5). Injections were optimized so that each surface was coupled with approximately the same amounts of myhsp60-H-2D^b or gp33-H-2D^b complexes (around 2500 RU). A blank surface was made by ethanolamine deactivation of the activated surface. Measurements were carried out at 20 °C in phosphate-buffered saline (PBS; pH 7.4) containing 0.005% (v/v) of Tween-20. All proteins were dialyzed against PBS (pH 7.4) prior to injection, and 0.005% of Tween-20 was added to protein solutions. For measurements of TCR binding to pMHC complexes, 10 μl of soluble TCR UZ3-4 were injected at 20, 50, 80, 110, 140, 170 and 300 μM at a flow-rate of 5 μl per minute at 20 °C. A control was carried out under the same conditions with injections of bovine serum albumin (5 mg ml) over the surfaces coated with pMHC complexes. Data were analyzed with the Biaevaluation 3.0 software. The dissociation constant was determined by fitting a hyperbolic curve to the RU *versus* TCR concentration plot (SigmaPlot 4.0).

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